Neoplasms and Related Disorders of Invertebrate and Lower Vertebrate Animals

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Neoplasms and Related Disorders of Invertebrate and Lower Vertebrate Animals

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NATIONAL CANCER INSTITUTE MONOGRAPHS

KENNETH M. ENDICOTT, *Director, National Cancer Institute*

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Conference ON

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Symposium Chairmen and Editors
CLYDE J. DAWE
JOHN C. HARSHBARGER

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INTRODUCTORY REMARKS

It is an honor and a pleasure to welcome you to the Smithsonian Institution for this Conference on Neoplasms on Related Disorders of Invertebrate and Lower Vertebrate Animals. This Symposium is sponsored by the Registry of Tumors in Lower Animals, which was established jointly by the National Cancer Institute and the Smithsonian Institution only a few years ago for the collection and study of neoplasms and related disorders in lower animals. We consider this a very important project and one that clearly overlaps areas traditionally devoted primarily to the field of medicine and areas devoted primarily to the field of biology.

Unfortunately, this region of biomedicine has been neglected. Biologists have devoted massive efforts to the increase of knowledge and understanding of living systems at all levels of complexity, while virtually ignoring the fact that every cell is liable to death. Diseased and otherwise abnormal specimens are ignored or discarded, sometimes contemptuously. I recall my distaste for preserving the occasional specimen of my own rare waterfowl collection that had died from some cause showing evidence of an unusual disease. When I did preserve such specimens, I had occasion to bring them to my late colleague Dr. Duran-Reynals at the Yale Medical School and was able to help him with more than one brain tumor in waterfowl.

Medical scientists, on the other hand, have vigorously studied diseases in humans and selected other mammals in an effort to find cures. In the process, they too have given little consideration to the possibility that pertinent information might be readily obtainable from studies in the simpler systems of the lower vertebrates and invertebrates.

The Smithsonian Institution has a tradition of pioneering in areas of neglect and is happy to cooperate with the National Cancer Institute on this project. This is the first international meeting ever convened specifically to consider tumors and tumorigenesis in lower animals. The material discussed and made available here will be strongly suggestive of the potential value of studies in lower animal pathology to our medical and environmental welfare. Let us hope the scientific world will be so challenged that this potential will be realized.

S. Dillon Ripley
Secretary
Smithsonian Institution
Washington, D.C. 20560

THE REGISTRY OF TUMORS IN LOWER ANIMALS

This Registry was established to facilitate the comparative study of tumorigenesis and related disorders in invertebrate and poikilothermic vertebrate animals. Comparative pathobiology often serves to reveal successful techniques that evolved in various ancestral groups as protective mechanisms against toxic materials, infectious agents, malnutrition, radiation, wounding, and other stressors. Comparative studies also illuminate basic elements of disease processes as they relate to life in general, and help to delineate the influence of refinements and specializations in the neural, circulatory, respiratory, and other systems in higher forms.

Many well-known basic principles of biology and pathology were first discovered in lower animals and plants by independent and imaginative scientists. For example, Bassi found that disease could be transmitted by a microbial agent (a fungus) during his study of muscardine in silkworms; Pasteur learned disease-fighting techniques by studying pébrine disease in silkworms; and Metchnikoff discovered phagocytosis in a crustacean.

Recognizing that coordinated pathobiological research will become increasingly valuable to biomedicine, the National Research Council's Section on Pathology established a subcommittee on comparative pathology. This committee met twice in 1963, under the chairmanship of Dr. Donald King, then of the University of Colorado Medical Center. Six projects were approved and received subsequent action, largely due to the efforts of the persons who introduced them. The proposals were:

I.—To encourage the organization of symposia on comparative pathology. The following symposia (listed chronologically) relating to this field have been held. It should be noted that the list below is provided primarily as a source of information. None of these symposia received financial aid from NRC-NAS, and several (1, 5, 6, and 9) were organized by individuals outside the Subcommittee.

1) Conference on Comparative Aspects of Reproductive Failure. Springer-Verlag, New York, 1967.
2) Defense Reactions in Invertebrates. Federation Proceedings 26: 1664–1715, 1967.
3) The Mammalian Oviduct. University of Chicago Press, 1969.
4) The Choice of Animal Models for Study of Diseases in Man. Federation Proceedings 28: January–February, 1969.
5) Differentiation and Defense Mechanisms in Lower Organisms. In Vitro, Publication of the Tissue Culture Association, Vol. III. Williams & Wilkins Co., Baltimore, 1968.
6) Comparative Morphology of Hematopoietic Neoplasms. National Cancer Institute Monograph 32, 1969.

7) Neoplasms and Related Disorders in Invertebrate and Lower Vertebrate Animals. National Cancer Institute Monograph 31 : 774 pp., 1969.
8) Conference on Comparative Mammalian Cytogenetics. Springer-Verlag, New York, 1969.
9) Biology of Amphibian Tumors. Springer-Verlag, New York, 1969.
10) Choice of Animal Models for the Study of Disease Processes in Man. Federation Proceedings 28 : 160–215, 1969.

II.—To design and conduct courses in comparative pathology. Two efforts have been made in this direction. Dr. Guido Majno, Dr. M. J. Karnovsky, and other members of the Department of Pathology of the Harvard Medical School conducted several summer courses in pathology for biologists, and Dr. Donald W. King has been conducting annual 2-week summer conferences in pathobiology at Aspen, Colorado, since 1964. These courses have been received with much enthusiasm, and have made great strides toward bringing biologists and medical scientists together.

III and IV.—To start a society and a journal for comparative patheology. The Society for Invertebrate Pathology was incorporated in 1967 with Dr. E. A. Steinhaus as President, Dr. A. K. Sparks as Vice-President, and Dr. A. M. Heimpel as Secretary-Treasurer. Dr. Sparks will be President from 1968–1970. The Society adopted the Journal of Invertebrate Pathology which was started by Dr. Steinhaus in 1959 as the Journal of Insect Pathology and was broadened in 1965 to include studies of diseases of all invertebrates.

V.—To reprint Elie Metchnikoff's "Lectures on the Comparative Pathology of Infection." This reprint is now available from Dover Publications, New York.

VI.—To support registries of comparative pathology and, particularly, to encourage the establishment of a registry of tumors in invertebrate and poikilothermic vertebrate animals. A registry of comparative pathology devoted primarily to warm-blooded vertebrates was established by the American Registry of Pathology of the Armed Forces Institute of Pathology in 1966.

Establishment of the Registry of Tumors in Lower Animals

The proposal to establish a Registry of Tumors in Lower Animals was actively supported in the Laboratory of Pathology of the National Cancer Institute by Dr. Harold F. Stewart, Chief; Dr. Thelma B. Dunn, Head of the Section on Cancer Induction and Pathogenesis; and Dr. Clyde J. Dawe and Dr. Mearl F. Stanton of Dr. Stewart's and Dr. Dunn's group. An Advisory Committee was established composed of Dr. Stewart, Dr. Stanton, Dr. Dawe; Dr. Frederik Bang of The Johns Hopkins University School of Hygiene and Public Health; Dr. Herbert Ratcliffe, Chief Pathologist of the Philadelphia Zoological Gardens; Dr. Donald Squires, Deputy Director of the Museum of Natural History, Smithsonian Institution; and Dr. Stanley Weitzman, Curator of the Smithsonian Institution's Division of Fishes.

Dr. Dawe and Dr. Stanton activated the Registry in 1965. Seventeen specimens were contributed from their personal collections, and brief descriptions of these specimens were included in the 1st Annual Activities Report. In July 1966, a contract was negotiated between the National Cancer Institute and the Smithsonian Institution for the continuation of the project, with Dr. Dawe as Project Officer and Dr. Squires as Principal Investigator. Dr. George E. Cantwell, who had been studying the effects of mammalian chemical carcinogens on invertebrates at the Insect Pathology Laboratory of the U.S. Department of Agriculture, took a year's leave of absence to become the Registry's first Director on September 1, 1966.

Dr. Cantwell organized the Registry at the Smithsonian Institution and sent announcements to scientists all over the world, stating the objectives of the Registry and soliciting specimens representing induced or naturally occurring neoplasms, preneoplastic lesions, or indeterminate lesions of presumptive neoplastic nature.

Operation of the Registry of Tumors in Lower Animals

Specimens are accepted alive or fixed, and a few have been submitted in frozen state. Frozen specimens are generally unsatisfactory for preparation of good histologic material but sometimes are useful as supplements to fixed specimens when an infectious agent must be sought. Frequently, prepared histologic sections or embedded tissue blocks have been submitted by investigators who have completed their personal studies, either published or unpublished. These are especially useful to other workers wishing to compare their findings with earlier observations.

Supporting information is an essential part of any acquisition that is to be useful. Therefore, it is always desirable that contributors provide data regarding scientific and common names of the diseased animal; the date and site of collection; the age, sex, and apparent state of health of the animal; the method of killing and preservation; and the size of the sample of undiseased animals among which the specimen was found. Photographs, references to publications, and information concerning environmental factors that may be related to the diseased state are particularly important. Though recognized with disappointing rarity, it is also important that one or more normal specimens of the same species accompany the pathological specimen. Without these, evaluation of gross and histologic changes must often rest upon an inadequate baseline.

Frequently, those who specialize in studies of a particular species or ecologic system are able to perceive or suspect environmental or genetic factors that no remote or inexperienced observer could possibly think of. Therefore, the contributor is in a unique position to advance cancer biology by applying his experience to the interpretation of the changes he has been astute enough to detect. For example, he may be able to correlate his observations with the exposure of his specimen to chemical or radioactive waste, sewage, contamination, herbicides or pesticides, or exceptional natural conditions, such as increased salinity or abnormal temperatures.

Whenever a specimen shows promise of usefulness as the subject of an experimental or field study, the Registry encourages the contributing scientist to extend his studies and report his findings. If technical assistance or collaborative aid is needed, the Director suggests sources of these from his knowledge of other workers in the pertinent disciplines. If the contributor does not wish to pursue study further, or is unable to, his permission is asked to allow others to make use of the specimens for investigations in greater depth. Published reports resulting from such studies must acknowledge the source of the material, but collaborative work and publication are the most acceptable approach and are earnestly sought.

Problematic lesions are often encountered among the accessions. Diagnostic opinions are then solicited from several experts on the animal involved, as well as from experts on neoplasia or whatever other process appears to be the appropriate disease homologue in other animals. Distinctions between neoplasms and exuberant inflammatory reactions, such as granulomas, are frequently difficult to make. The literature seems to be replete with reports of granulomas, hyperplastic conditions, and repair processes that have been mistaken for neoplasms. Serving as a center not only for collection but more importantly for analysis and reevaluation of diseases processes, the Registry must revise the classification of specific lesions in pace with accumulation of information and the evolution of improved understanding.

On April 15, 1969, the Registry listed 241 accessions distributed among classes or phyla as shown in table 1. The total number of specimens approaches 1,000, since multiple type-specimens are frequently submitted.

Table 1.—Distribution of accessions among various classes and phyla

Reptilia	23	Echinodermata	2
Amphibia	19	Annelida	6
Pisces	71	Platyhelminthes	5
Arthropoda	53	Coelenterata	6
Mollusca	51		241

About 30% of the specimens contain lesions which satisfy criteria generally acceptable for mammalian neoplasms, and all these specimens belong to the first 5 groups of table 1. The remaining specimens show a variety of disorders, including infectious granulomas and other infectious lesions, as well as parasitic infestations, developmental and teratoid anomalies, and responses to noxious agents of unknown nature. Contributors have been strongly encouraged to submit representative non-neoplastic lesions to the collection for comparison with neoplastic changes.

A nearly complete library of the literature on neoplasms of invertebrates has been collected by the Registry, and new references are reported annually in the Activities Report. Copies of the reference list are available from the Registry upon request. Literature pertaining to neoplasms in poikilothermic vertebrates is also being acquired, with the aim of achieving a complete collection in this area as well.

Experimental activities, while subordinate to the service role of the Registry, are being undertaken in two general areas: 1) tumor induction, which includes studying the effects of various mammalian carcinogens on cold-blooded animals, and 2) neoplasia in populations that have a sufficiently high natural prevalence of tumors to make study feasible. Such populations may conceivably result from carcinogen-contaminated environments, from development of genetic pools that predispose toward neoplastic disease, from nutritional deficiency (as with goitrous fish whose diets are iodine-deficient), from viral infection, from aging, or from other factors.

Perspectives of the Registry of Tumors in Lower Animals

The current activities of the Registry will be continued and expanded to increase the body of knowledge on disorders of growth in lower animals and the materials needed for advanced study. Every effort will be made to determine how this knowledge may relate to knowledge of neoplastic diseases in higher animals. Examples already are known where interesting parallels can be drawn. Herpesviruses are widely distributed in nature, and have been found in association with the Lucké renal neoplasms of frogs, Marek's disease (lymphoma) of chickens, and Burkitt's lymphoma of man. When the relationship between virus and neoplasm becomes clear in one of these systems, the way may be opened toward clarifying the role of virus in the other two.

Papillomas may be cited as another example. They are found in a wide variety of animals including man, and in many species are known to be caused by viruses of the papova group. They can also be induced in many laboratory animals by a number of crude and purified chemicals. Among the fishes, as documented elsewhere in this Symposium, papillomas are quite common and may affect certain populations to the exclusion of others. Thus there is the possibility that papillomas may serve as indicators of either viral or chemical carcinogens in the aquatic environment. Investigations of papilloma incidences in relation to domestic sewage outflows and industrial waste disposal are much needed.

Mechanisms by which animals are protected from neoplasms have long been sought. At least three devices that may be peculiar to lower animals have been noted by participants in this Symposium: 1) Insects may recognize incipient tumor cells and encapsulate them before they have an opportunity for full expression; 2) some invertebrates, such as planaria, are capable of autectomy of diseased parts, and it has been suggested that this mechanism might be used to eliminate neoplasms; and 3) mollusks contain substances that appear to inhibit growth of certain transplantable tumors of higher animals.

All of these possible protective mechanisms deserve further investigation, for at this time it appears either that invertebrate neoplasms occur only rarely or that investigators have only rarely been able to find and recognize them.

Other examples of the biomedical research potential offered by the lower organisms receive emphasis throughout this Monograph. Numberless opportunities will be evident to biologists who work with lower animals and to cancer researchers who need special biologic systems for the advancement of their investigations. An objective of the Registry is to support these investigations by serving as a center of information and specimen reference material.

John C. Harshbarger, *Director*
Registry of Tumors in Lower Animals
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Phylogeny and Oncogeny

CLYDE J. DAWE,[1] *Laboratory of Pathology, National Cancer Institute,*[2] *Bethesda, Maryland 20014*

Cancer

In tiniest fragment of life-code you lie
Mal-structure of triplet that brings all to die.
    Mite monsters! What perverse distortions arise!
    And magnified transform to dew-glistening eyes.
Bernard L. Strehler (*1*)

Among the most promising endeavors in the biological sciences today is the intensified drive by molecular biologists to discover control mechanisms of cell replication and differentiation. It is implicit, as the verse by Strehler reflects, that part and parcel of these pending discoveries will come understanding of the defective controls underlying the disorders grouped sophistically as neoplasia. The secrets of neoplasia are, collectively, the Grail that will reward beknighted science when it has proved its worthiness on the exacting jousting grounds of molecular biology.

Is this a well-supported assumption? It is if one has solidly in hand the characteristics of the disorders in question. If one knows these characteristics at the successive interlocking levels above the molecular level, it matters little what one calls the disorders; it should be possible to find the molecular bases for these characteristics and to assemble a morbid anatomy of disease from molecular through cellular and organismal levels.

However, if one does not have the supramolecular characteristics of disorders in hand, the triumphs of molecular biology can lead only to the compilation of "orphan" molecules and molecular interactions in search of diseases. Viruses in search of diseases (orphan viruses) exemplify the problem at a slightly higher level.

---

[1] I think Mr. Gebhard Gsell, Laboratory of Pathology, National Cancer Institute for the photographic illustrations. The diagrams for text-figures 2, 3, and 9 were done by Mrs. G. Nicholson, for text-figure 6 by Mrs. Elva Sawyer, and for text-figure 7 by Miss Marian Gundy, all of the Medical Illustrations Section, Medical Arts and Photography Branch, Division of Research Services, National Institutes of Health.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

There exists a gap, phylogenetically viewed, in our knowledge of neoplasms and related disorders among the animals proximal to the fishes in time of evolutionary origin. If this gap is not soon filled, the advantages offered by study of these animals cannot accrue to cancer research until long after the time when they might have been exploited to the fullest. This Monograph presents evidence to support this contention; *e.g.*, it contains reports of sophisticated biochemical researches relating to helminth diseases (*2, 3*), whose characteristics are still quite vaguely defined and which may or may not eventually be shown to have features in common with neoplasms of vertebrates. Substances that seem able to inhibit transplanted mammalian neoplasms are reported (*4*) from mollusks, which on one hand have been claimed not to suffer from neoplasia, and on the other are the subject (*5–8*) of case reports of possibly neoplastic diseases only recently brought to light, and of a review (*9*) of other cases now almost ancient in the literature. The comparability of melanotic tumors of *Drosophila* with any neoplasms known in vertebrates remains in dispute (*10, 11*), after some 50 years of analyses that have still not yielded many of the needed facts relevant to the question. Similarly, certain lesions in cockroaches have been known and discussed in the literature of insect tumors for years (*12–17*), without a frank confrontation of the question whether these lesions may be more comparable to granulomas than to neoplasms of higher animals. Only in the last few years have transplantable lines of insect cells been developed (*18, 19*), and ironically some of the evidence coming from these works, instead of supporting the concept that the serially transplanted cells are neoplastic, seems rather to overthrow the long-held view that continuous serial transplantability is indisputable evidence of neoplasia.

Why has it taken so long to characterize disorders of growth and form in the lower phyla, even at supramolecular levels? Certainly it is not because of a late start. Comparative oncology had its beginnings before the cell concept of disease was established. In 1802 (*20*), what was apparently the first society for cancer research proposed the following question among 13 that were deemed of critical importance:

> Query 10th.—Are brute creatures subject to any disease resembling cancer in the human body? It is not at present known whether brute creatures are subject to cancer, though some of their diseases have a very suspicious appearance. When this question is decided, we may inquire what class of animals is chiefly subject to cancer; the wild or the domesticated; the carnivorous or the gramnivorous; those which do, or those which do not, chew the cud. This investigation may lead to much philosophical amusement and useful information; particularly it may tell us how far the prevalence or frequency of cancer may depend upon the manners and habits of life. . . .

But advances came slowly in earlier days. Thirty-six years passed before Johannes Müller (*21*), in 1838, brought the microscope to bear and recognized the cellular composition of human cancers; 20 more years went by before the veterinarian Leblanc (*22*) affirmed that growths in domestic animals had a cellular composition similar to cancers in man. We are thus marking the 110th anniversary of the beginning of comparative oncology at the cellular level. Biologists will note a useful chronologic reference point in Darwin's "On the Origin of Species" (*23*), also published just 110 years ago.

Despite Leblanc's unambiguous stand and his plea to use medical pathologists' methods to study tumors of animals other than man, it was nearly another 50 years before Bashford (*24*) announced:

> The first authentic case of a malignant new growth outside of the mammalia, viz. in a trout, was brought to the notice of the Cancer Research Fund in a letter and enclosures dated March 16th, 1903, from Mr. J. A. Gilruth, of the Government Veterinary Department, New Zealand. Indebtedness for much valuable assistance is also acknowledged to Captain Le Poer French, Superintendent of Billingsgate Market, and to Dr. Carrington Purviss, who kindly sent two specimens of carcinoma, obtained in 1888–89, from trout at the Solway Fisheries, Scotland.
>
> The unequivocal nature of the microscopical preparations of adenocarcinoma in fish obviates completely the objection that might be raised, were sarcoma (so often with difficulty distinguished from infective granuloma) alone recorded in this class.

Reviews in this Monograph (*9*, *25*) may suggest some reservations concerning Bashford's first sentence quoted above, since neoplasms in fish had been reported much earlier than 1904 or 1888. Besides, Ryder (*26*) had reported microscopic identification of a tumor, albeit noninvasive, in an oyster in 1887.[3] While Ryder's histologic description leaves the type cell of origin of his tumor quite enigmatic, he made a statement that leaves no doubt of his concurrence with the thought expressed in the Edinburgh (1802) questionnaire:

> It is also of great interest as proving that such pathological growths may be developed in the mollusca, thus showing that even in the invertebrata there may be morbid proliferations of certain tissues which simulate in certain respects those observed to occur in man and the higher types of vertebrates, in which they become very dangerous and painful in character, as in the case of cancer.

The years since the publication of Bashford's paper at the turn of the century have seen massive accumulations of information reaffirming that neoplasms occur in animals below man and are often similar to those in man. With accumulation of experience, it also became clearly evident that animal neoplasms nearly always differ in one or more aspects from their closest counterparts in man. This came as no great shock to those thoroughly familiar with cancer clinically, for they already knew that no two neoplasms in man look or behave exactly alike either. And yet the differences between animal tumors and human tumors received such emphasis that comparative oncologists have repeatedly been called on to defend the rationale of their interests. Referring to the era of cancer research extending even into the second quarter of this century, Dunn (*27*) in 1962 remarked:

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[3] Presented at a meeting of the Academy of Natural Sciences of Philadelphia. Ryder's specimen was given to him by Professor J. Leidy, who in 1851 had reported, before the same Academy, attempts to transplant subcutaneously human tumors to frogs. The Leidy Laboratories of Biology, University of Pennsylvania, are a memorial to Dr. Leidy. It is also of historical interest that at the same meeting at which Ryder presented his molluscan tumor, William Osler, M.D., was admitted to membership of the Academy and the Rev. Henry C. McCook, D.D., presided.

> It seems incredible today that for many years some physicians and biologists were unconvinced and contemptuously referred to tumors in mice as "pseudoplasms." The National Cancer Institute was called the National Mouse Cancer Institute.

Before finding too much amusement in this retrospective glance, it is well to note that opinion is still sharply divided concerning the "authenticity" of neoplasms in those phyla with which this Symposium is concerned (excepting neoplasms of fish, a class of vertebrate). Even among the participants, there are those who question the occurrence of "true" neoplastic disease among invertebrate animals.

Very likely, some accept the universality of neoplasms among all metazoan animals as a foregone conclusion and see little reason to be further concerned. But I hope most participants are aware that the available hard facts concerning anomalies of growth and form in invertebrates are few. What is more, a great deal of thoughtful work needs to be done before the significance of what *is* known can be clearly understood.

To here, I have observed that a gap exists in knowledge of the biology of neoplasms of invertebrates. I have urged filling this gap and have suggested that the logical way to do this is from the bottom up; that is, from organismal, organ, tissue, and cellular levels upward so that information already arriving at the molecular level will find underpinnings to rest on. Why is it important to fill the gap at all, at any level? Is it merely for the "philosophical amusement" mentioned in the Edinburgh questionnaire? Is it simply to fill in the empty areas where evolutionary theory, already well-supported, suffers some slight neglect? Is it to perform the ritual, depicted in Orozco's mural (*28*), of delivering dead academicism of a mortar board-capped fetus that will grow into a new academy to mother new mummified fetuses like itself? Or might it be that we are helplessly devoted to a pastime of "histophilately," a marvelously apt term coined (*29*) to describe the activity of collecting histologic sections for the sake of a prestigious collection, not for the sake of the messages those sections have the power to convey?

None of these reasons is adequate. The reason is that phenomena must be known before the mechanisms of these phenomena can be studied and before analyses can be made of relationships of various phenomena to each other. This is another way of expressing the poetic comment of Hughlings Jackson (*30*): "The study of things caused must precede the study of causes of things."

The Eternal Surveyor (*31*) may carry a low status rating today, but surely some amount of surveying *must* be done before hypotheses can be conceived for testing. Darwinian evolution is perhaps the outstanding example of a hypothesis conceived of a survey (voyage of the *Beagle*), though assisted by arithmetical logic (the Malthusian *Principles of Population*) (*32*).

Provided with some observations, the investigator's job is to construct a hypothesis or several alternative hypotheses that are susceptible to test. As in other branches of sciences, this approach is elementary in comparative oncology. For example [*see* (*33*) for review], after it became known that workers exposed to 2-naphthylamine had an unusually high incidence of bladder cancer, several species of mammals were experimentally exposed to this compound. It was found that dogs, but not other species, developed bladder cancers. This required a hypothesis to explain why dogs and men, but not many other mammals, are susceptible to the carcinogenic effects of 2-naphthylamine. Among several possible hypotheses to explain this, two were most attractive: 1) Dogs and men might lack a biochemical process for inactivating 2-naphthylamine; or 2) dogs and men might have a biochemical process that converts 2-naphthylamine to an active carcinogen. Laboratory experiments showed the first alternative could be excluded. Although the entire biochemical mechanism is still not clear, it is now evident that, in dogs and men, 2-napththylamine is converted to several active carcinogenic compounds able to induce bladder cancers in other species (mice) not susceptible to the effects of 2-naphthylamine itself.

From evidence presented in this Monograph, it seems that we are already in a position to use the method of strong inference in a similar situation concerning liver carcinogenesis in salmonid fishes. Hepatomas occur in rainbow trout given aflatoxin in their food, but not in coho salmon given similar doses by the same route (*34*). Does the coho salmon have an inactivating or an eliminative mechanism, or does the rainbow trout have an activating mechanism or a failure in its eliminative mechanism? At least four alternative hypotheses, not mutually exclusive, are susceptible to test in the next experimental steps probing this system.

The information available regarding neoplasms of invertebrates has long been so scant that hypotheses for testing have been limited to those that postulate one or another protective mechanism that would make it impossible or highly improbable for invertebrates to manifest neoplasia. At least three investigators (*4*,*35*,*36*) have looked for antitumor or growth-inhibiting substances in invertebrate animals, and thus secured some evidence possibly relevant to the hypothesis that these animals may have special protective mechanisms. All three investigators reported positive findings, but the possession of substances inhibiting growth of transplanted neoplasms of vertebrates need not explain a low incidence or exclude a high incidence of neoplasms among the very animals producing the inhibitors. Every defense has its weak points, and local breakdowns in the availability or effectiveness of putative tumor inhibitors could allow neoplasms to arise. Also, there is no direct evidence that molluscan antitumor extracts are effective protectors against oncogenesis in mollusks themselves, whether of the same or of related species. Still further, the history of therapeutic trials in experimental animals cautions us against too-ready acceptance of the molluscan extracts as specific antitumor agents.

The phylogenetic tree itself provides cancer research with a "conditional inductive tree" or "logical tree" of the type that invites use of the Baconian method of strong inference (*31*). But to use the phylogenetic tree as a framework requires knowledge of the facts, and the tree is being "climbed backward" by scientists, whereas, of course, it developed evolutionarily in the opposite direction. Nevertheless, by noting changes that occur proximal to the divergent limbs at each bifurcation, it should be possible to look backward and construct hypotheses for testing.

This has been done, not only in the past, but in the organization of this Symposium which is constructed on the phylogenetic framework. Our starting point is the Class Pisces of the Vertebrata, since here we are on a firm limb bearing abundant undisputed evidence (*25*) of the occurrence of neoplasms biologically and morphologically similar to those of the warm-blooded vertebrates. Troubles begin as soon as one descends below the elasmobranchs. No neoplasms have previously been reported in the cyclostomes, which include the hagfishes and the lampreys. Is this because they don't occur, or only because they have not been found? There is no absolute way to answer this, but whenever a positive example is found, it goes a long way toward making this question obsolete, and introduces newer, more forceful questions. The Registry of Tumors in Lower Animals of the Smithsonian Institution has been fortunate in receiving from J. H. Howell (*37*) an adult sea lamprey bearing multiple tumors (fig. 1). Histologically these tumors resemble neurilemomas of other vertebrate species (fig. 2). This single specimen, which deserves to be described in full detail in the future, does not provide a sufficient base on which to construct a hypothesis, but it does place constraints on any hypothesis founded on the previous lack of knowledge of tumors of vertebrates below the elasmobranchs. It should also stimulate search for additional positive information; and it pushes the "phylogenetic termination point" of tumors below the elasmobranchs, where Teutschländer placed it (*38*). Evidence presented by several participants in this Symposium moves this point still further, well into the invertebrate phyla. One cannot foresee where the termination point, if there is one, will come to lie.

Closely bound up with this question of moving proximally along the phylogenetic tree in search of neoplastic phenomena is the problem of identifying neoplasms. One may fail to find neoplasms because they are not "available," or because they are not recognized. Neoplasms may not be available for many reasons: 1) They might not indeed occur and, if so, they never will be found; 2) they might be destroyed, along with their hosts, by predatory natural enemies that take advantage of any slight debilitation of the prey; 3) they might occur only in age ranges rarely reached under natural conditions; or 4) they might be eliminated or "self-cured" by special mechanisms, such as autectomy, available to certain animals. All of these questions can be classed as "trivial," for they can obviously be attacked in a simple, practical way. By bringing animals into the laboratory where they can be closely observed and protected from environmental depredations, one can answer these questions. Andervont and Dunn (*39*), studying captive wild mice in the laboratory found the mice developed many "spontaneous" neoplasms, thereby disproving the hypothesis that mice directly of wild heritage are not susceptible to cancer.

But the question of how to recognize neoplasms when confronted with them is not so trivial and should be considered as one of the knottiest problems of all. How can one say yes or no about a qualitative test whose endpoint is not clear? I am not thinking here of the problem of defining neoplasia constitutively—a problem that is of even greater fundamental importance—but of that of identifying or recognizing even *presumptive* neoplasms. The net must be fine enough to catch at least the grossest specimens, else it will miss all.

There is a tendency to think that neoplasms should present themselves as lumps or bumps. Though many neoplasms fit this image, the fact is that most lumps and bumps, even in mammals, come from infections, parasitic infestations, injuries, and other non-neoplastic causes. Every lump or bump is therefore not a tumor (neoplasm), but has to be examined with this possibility in mind. Neoplasia has many faces, as recognized by the predecessors (*40*) of Leblanc:

> Carcinoma is indurated by inflammation, at times ulcerated, from bright to nearly dark red in color, oozing blood, remarkably non-cohesive, enveloping within itself small veins and even hairs. A mad disease.

If the manifestations of neoplasia are so protean for mammals, how much more difficult must it be to recognize them among invertebrates, which have so many traits at variance with those of mammals? One obstacle to general acceptance of organic evolutionary theory was the difficulty of crossing some of the wide gaps presented by incredible anatomic differences between the phyla. Darwin, in Recapitulation and Conclusion (*23*), commented: "But the chief cause of our natural unwillingness to admit that one species has given birth to other and distinct species, is that we are always slow in admitting great changes of which we do not see the steps."

Long after the first publication of *On The Origin of Species*, D'Arcy Wentworth Thompson (*41*), in his classic work *On Growth and Form*, elaborated on Darwin's thought. Referring to the difficulties in establishing linkages between evolutionary forms, he wrote:

> But the larger and at first sight simpler questions remain unanswered; for eighty years of Darwinian evolution has not taught us how birds descended from reptiles, mammals from earlier quadrupeds, quadrupeds from fishes, nor vertebrates from invertebrate stock. The invertebrates themselves involve the self-same difficulties, so that we do not know the origin of the echinoderms, of the molluscs, of the coelenterates, nor of one group of

> protozoa from another. The difficulty is not aways quite the same. We may fail to find the actual links between the vertebrate groups, but yet their resemblance and their relationship, real though indefinable, are plain to see; there are gaps between the groups, but we can see, so to speak, across the gap. On the other hand, the breach between vertebrate and invertebrate, worm and coelenterate, coelenterate and protozoan, is in each case of another order, and is so wide that we cannot see across the intervening gap at all . . . . In natural history Cuvier's "types" may not be perfectly chosen nor numerous enough, but *types* they are; and to seek for stepping-stones across the gaps between is to seek in vain, for ever . . . .
>
> This is no argument against the theory of evolutionary descent. It merely states that formal resemblance, which we depend on as our trusty guide to the affinities of animals within certain bounds or grades of kinship and propinquity, ceases in certain other cases to serve us, because in certain circumstances it ceases to exist.

So it may be with neoplasia in relation to phylogeny. The differences between neoplasms of invertebrates and of vertebrates may be so great that we fail to recognize their nature and kinship.

There are some examples that support this contention. One of these is concerned not with neoplasia, but with inflammation. Scharrer (*16*) noted that it could be argued that inflammation cannot occur in certain invertebrates, inasmuch as an essential part of inflammation is the blood vascular response and many invertebrates have no clearly developed blood vascular system. Yet it seems unreasonable to exclude from consideration those components of inflammation that do occur in invertebrates, such as the accumulation of interstitial fluid (edema) and the immigration of phagocytic cells into injured areas, sometimes followed by encapsulation of the noxious agent by the phagocytes. The classic works of Metchnikoff (*42*), identifying the conversion of fixed mesenchymal cells to migratory phagocytes in Daphnia and in starfish larvae, are the basis of our concepts of the phylogenetic origins of blood cells, as well as of their ontogenetic origin from mesenchymal cells in embryos.

Directly relating to the problem of recognizing neoplasms among invertebrates is the restudy by Squires (*43*) of an unusual coral specimen that had been acquired by the National Museum of Natural History a half century earlier. Three abnormal corallites, previously thought to represent a foreign species attached as an epizoite to a colony of *Madrepora kauiensis*, were reinterpreted as anomalous growths stemming from *M. kauiensis* and having certain features often associated with neoplasms of higher animals. This illustrates that it may be difficult to sort out problems of species identification from those of neoplasm identification. If this example holds more general implications, then a wealth of information pertinent to our understanding of neoplasia may be hidden behind little more than a set of too rigidly fixed thought patterns.

Lenhoff *et al.* (*44*) in this Monograph adds credence to this possibility by comparing the dominant behavior of a mutant hydra's cells, transplanted to a normal parent-line host, with that of a neoplasm transplanted to a compatible host. Speciation has been compared with tumor genesis before, by Huxley (*45*), Hauschka (*46*), and many others. An experienced pathologist of my acquaintance had a playful custom of classifying tumors as different breeds of cats; some he nicknamed lions and tigers, others pussycats. But Lenhoff *et al.* (*44*) have provided a tangible example in which the fine line between speciation processes and oncogenic processes is in fact hard to discern. In an asexually reproducing animal, how would one distinguish between speciation and tumor genesis if a dominant somatic mutation occurred in a totipotential cell such as the interstitial cell of hydra is supposed to be? Since the observations of Lenhoff *et al.* dealt with a sexual mutant rather than a somatic one, one cannot answer this question. But it may be possible to induce similar mutants to occur in asexual hydra by use of physical or chemical mutagenic agents such as X ray or various alkylating agents.

In hydra, there is a phenomenon well described by Brien (*47*) and long familiar to hydra specialists. When *Hydra pirardi* is cultured at low temperatures (8°C), it goes into sexual phase and develops testes and ovaries. If the temperature is then raised to 19°C, the testes involute, and from their previous locations develop sprouts resembling incomplete heads or disorganized parts of hydra. To those who are familiar with teratocarcinomas of the testis in mammals, this sequence of events recalls that there is a higher incidence of teratocarcinomas in undescended testes than in intrascrotal testes where a lower temperature is maintained. Can the anomalus sprouts from involuted testes of hydra represent an equivalent of teratocarcinoma in man? In discussing this with hydra specialists, the latter have been quick to remind me that in some hydra the temperature-sex phase relationship is just the reverse of that for *H. pirardi.* This does not seem to invalidate the analogy, since a temperature adaptive response is still involved. This system perhaps offers an opportunity for hydra investigators to carry out studies comparable to those of Stevens (*48*) and Kleinsmith and Pierce (*49*) on teratocarcinogenesis in the testes of mice.

Still another way to emphasize the problem of recognition of neoplasms in less highly organized animals is to pose the following theoretical question about a theoretical animal: If the simplest marine metazoan were simply an obligatory aggregate of like cells that had evolved from some nonaggregated protozoan ancestor, and one clone within this aggregate "learned" how to get along independently as dispersed single cells in the marine environment, would this newly independent lineage of cells properly be called a new species of protozoan (derived through "reversed evolution") or a neoplasm? Though the situation posed is artificial, in the primitive sponges there do exist animals of nearly this degree of simplicity, and studies on the aggregation phenomenon in sponges (*50*) might lead to information relevant to neoplasia if ways can be found to make sponge cells irreversibly unable to reaggregate, yet able to survive and propagate as dispersed cells.

LOWER ANIMAL NEOPLASMS

Oncogeny in Relation to the Phylogenetic Origin of Immune Mechanisms

The phylogenetic origins of antibody production, cellular immunity of the homograft rejection type, and interferon production are subjects in which remarkable advances are currently being made. Among these advances, perhaps most relevant to oncogenesis are the findings pertaining to cellular immune mechanisms, for a large amount of evidence has been gathered to show that a defective thymic system can result in increased incidence of neoplasms induced by viral agents (*51–59*). Much less evidence is available that this system can influence chemically induced tumors (*60–62*).

In this Monograph, Good (*63*) reviews and presents information concerning his investigations and theories on the phylogeny of cellular immune mechanisms and the manner in which this relates to what may be called the phylogeny of neoplasia. He believes that effective thymic systems did not appear evolutionarily until lampreys or their more primitive forebears came on the scene, though the rudiments of this system may barely be detected in the still more primordial hagfishes. He has postulated that the thymic system serves as a surveillance and protective mechanism to detect and eliminate aberrant cell populations that would otherwise progress to overt neoplasia. This is a useful concept because it can be and has been subjected to test by observation of tumor incidences in animals in which the thymic systems have been ablated. However, such experiments do not exclude the possible existence of alternative or supplementary mechanisms of protection against neoplasms. Again, it becomes essential to know some facts about neoplasms among animals that lack, or appear to lack, cellular immune mechanisms. We are in the embarrassing position of having too little knowledge of neoplasms among animals that lack thymic systems, as well as too little knowledge of possible alternative or more primitive mechanisms of protection against foreign or aberrant cells. Hence, we are momentarily in a poor position to exclude alternative hypotheses. From existing observations (*25*), it *appears* that the prevalence of neoplasms drops off sharply as one passes from the elasmobranchs downward through the cyclostomes, the cephalochordates, the hemichordates, and into the tunicates. This drop-off appears to run approximately parallel to the disappearance of the thymic immune systems as studied by Good and others. This apparent parallelism could be explained by a number of hypotheses: 1) The lowest vertebrates and the prevertebrates might have an unusually low rate of incidence of aberrant cell populations (incipient neoplasms), so that overt neoplasms would be infrequent despite lack of an immune defense mechanism; 2) these same groups might have a rate of incidence of aberrant cell populations equal to that of higher vertebrates, but counteracted by some paleoprotective mechanism that suppresses the incipient neoplasms more effectively than the neoprotective mechanisms recognized in the form of thymic systems; or 3) more probable than either of the above possibilities is that animals of the lower vertebrates and prevertebrates would have variable incidence rates of incipient neoplasia, but would be protected by a combination of paleoprotective and neoprotective mechanisms offsetting these incidences.

After all, it is rather difficult to suppose that the hagfishes, with a set of organ, tissue, and cell systems nearly or equally as complex as those of the lampreys, should be able to fend off aberrant cell populations purely by means of some primitive system and equally as well as the lamprey, equipped with a presumably more effective neomechanism. One suspects that some overlap might exist, as Anfinsen (*64*) has noted at biochemical levels in phosphagens. In the tunicates, Anfinsen observed that both arginine phosphate (an invertebrate phosphagen) and creatine phosphate (a typically vertebrate phosphagen) are present. Thus the members of this subphylum lying between invertebrates and vertebrates have it both ways. Perhaps the prevertebrates and lower vertebrates also have it both ways with respect to cellular immune mechanisms; if so, these groups are particularly valuable for comparative studies of neoplasia in relation to immune mechanisms. Until now, only one neoplasm has been recorded in a lamprey (*37*), none in hagfishes, one in a cephalochordate (amphioxus) (*65*), none in hemichordates, and none of reasonable authenticity in tunicates. While little is known about prevalence of overt neoplasms in these groups, nothing is known of incipient neoplasms or aberrant cell populations in them. If one turns to the echinoderms, the lesions described by Fontaine (*66*) in a species of brittle star may prove to be of interest as incipient neoplasms, although these lesions will require more complete characterization before they are understood.

That invertebrate animals do have ways of recognizing and eliminating foreign antigens and cells has long been known (*67*). Whether a memory phenomenon also exists has not been established, and Cooper's paper (*68*) in this Monograph describes an attack on the problem through graft rejection studies with earthworms. It has been shown that cockroaches eliminate grafts of vertebrate neoplastic cells and cells from continuous culture lines of cold-blooded vertebrate origin (*69*). The elimination process was accompanied by a local cellular (hemocytic) response effecting encapsulation of the grafts, but whether a specific immune response was involved was not established. No accelerated rejection after a previous graft was observed, and the rejection might well have been brought about entirely by physical factors aided by nutritional deficiencies suffered by vertebrates' cells in an insect's internal milieu.

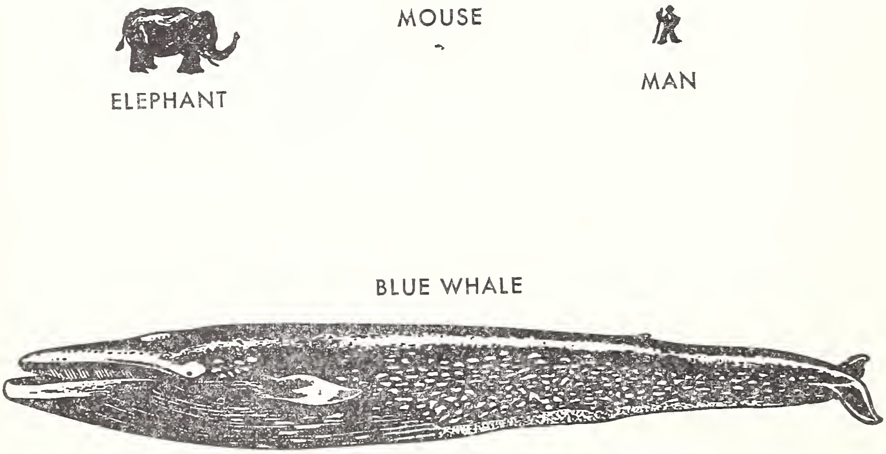
The phenomenon of autectomy may prove of interest to immunologists. Certain invertebrates, *e.g.*, helminths, can shed parts infested with parasites or otherwise diseased (*67*). These parts are then replaced through regenerative processes and the animal is restored to health. Two independent reports of spontaneously occurring anomalies of growth and form in flatworms have recently been made (*70, 71*). These deformities were eliminated by autectomy in some cases, but no information exists as to whether this involved recognition of autochthonous aberrant cell populations. Investigations of the events leading to autectomy may reveal whether there are any phylogenetic relationships between autectomy and graft rejection processes.

Systems Control and Neoplasia

Related to the immunologic concept of control of tumor genesis is the concept of intrinsic systems controls. These two concepts are not necessarily antithetical and should be considered supplementary. The immunologic concept may be compared with the development of inspection methods for detecting defective parts in a machine, eliminating them, and replacing them with better parts. The intrinsic systems control concept compares with the engineering practice of designing and constructing the original parts in such a balanced and coordinated way that there is no weak part that would break down and doom the whole machine to failure at an early time. A way to illustrate this concept is to pose one of the riddles in cancer research that has not been solved for vertebrate animals and which must surely be dealt with in investigations of invertebrate animals.

This riddle asks: Why don't extremely large animals develop neoplasms with a much higher incidence than very small ones since the cell population at risk is greater by several orders of magnitude? Implicit in this question is the assumption that the probability of origin of an aberrant, neoplastic cell lineage is the same per unit of cell population, regardless of species or cell type concerned. While this assumption is probably not valid, the question when turned around is equally enigmatic, for it then asks: Why are some species much more resistant to "spontaneous" neoplasia of a certain cell type than others, on a cell-at-risk basis?

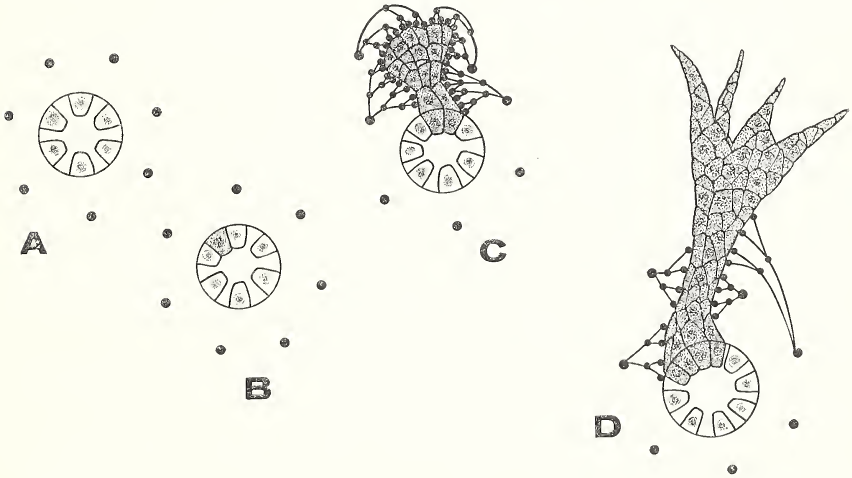
As an extreme example, text-figure 1 depicts the relative sizes of a blue whale, an elephant, a man, and a mouse. If one takes the weight of the mouse as 30 g and that of the blue whale as 100 tons, the whale is equivalent to 3,030,303 mice. Then if one accounts for differences of lifespan (65 years



TEXT-FIGURE 1.—Relative sizes of man, elephant, mouse, and blue whale. *See* text for discussion of relationship between size and risk to neoplasia.

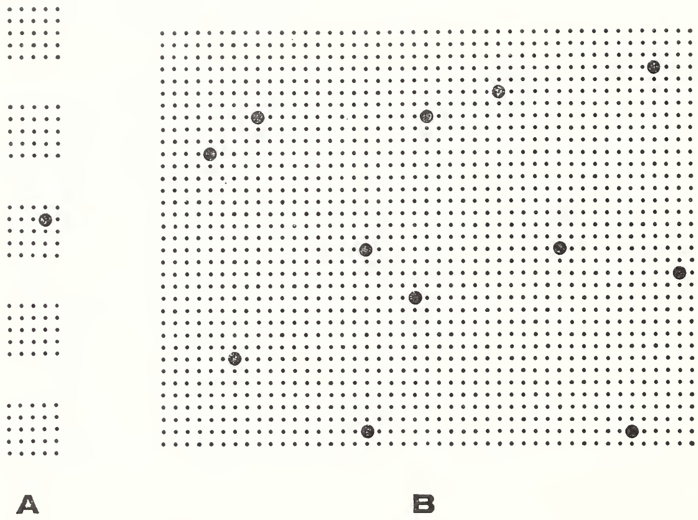
for the blue whale, 3 years for the mouse), the ratio of weight-year units per whale to weight-year units per mouse is about 66,670,000. Does this mean the whale has a 6.7 × 10^7 greater probability of developing a neoplasm than a mouse? Surely this is not the fact, for in a study of neoplasia among untreated wild mice kept under laboratory observation, Andervont and Dunn (*39*) found 121 neoplasms in 98 animals of a total of 225. On a weight-year basis, every blue whale should have developed about 20,000 neoplasms before completing its first year of life, as blue whales have been found to weigh about 8000 pounds at birth. As all are aware, the incidence of neoplasia is not a simple function of protoplasm at risk per unit time.

Following this same grossly oversimplified argument, it would appear than any defense system based on a constant breakthrough rate of aberrant cell populations could not adequately explain the ability of large animals to reach reproductive age without neoplasia intervening. Text-figure 2 depicts a defense system based on cellular immune mechanisms. It is basically a "cell-to-cell" secondary defense, as opposed to a primary "constitutional" defense. In *B*, an aberrant cell has appeared, but remains *in situ.* In *C*, aberrant cells have moved beyond the basement membrane and have started to invade surrounding tissue. However, the lymphoid cell response, involving blastogenic transformation and proliferation by the policing lymphocytes, has succeeded in containing the aberrant population and is in the process of aborting overt carcinogenesis. In *D*, the lymphoid cell response has been inadequate, and breakthrough of an early neoplasm has occurred.



TEXT-FIGURE 2.—Defense against genesis of neoplasms by cellular immune mechanisms. *A:* Normal gland cells (*unstippled*) are under surveillance of lymphoid cells (*black spots*) in peripheral ring. *B:* A neoplastic gland cell (*stippled*) has appeared, but remains *in situ. C:* Neoplastic cells (*stippled*) have proliferated, but are being eliminated by lymphoid cells, which have increased in response to tumor antigens. *D:* Neoplastic cells have outdistanced the cellular defense, resulting in neoplastic breakthrough.

If it is arbitrarily assumed (text-fig. 3) that a breakthrough occurs at a rate of 1 per 125 potential carcinogenic cell units per unit time and each animal has 25 such units, a neoplasm will occur once in every 5 animals per unit time. However, in a larger animal, but one with a "police system" of the same efficiency, breakthroughs will occur in absolute numbers increased in proportion to the increased number of units defended. In other words, an animal with 1500 units to defend should develop about 12 neoplasms per unit time.



TEXT-FIGURE 3.—Theoretical influence that size of organ would have on probability of neoplastic development, if one assumes equal rates of appearance of aberrant populations and equal rates of defense-mechanism failures, on a cell popuation basis. *See* text for discussion.

Returning now to the comparison between mouse and whale, we should expect the whale to develop neoplasia about 3 × 10^6 times more often than the mouse, per lifespan. This would hardly allow whales to exist today. Of course, it might be supposed that whales have a relatively more heavily populated "police" system than mice. But it could hardly be multiplied by a factor of 10^6, for the lymphatic system of the whale could not be increased by such a factor relative to total body mass.

There are many possible mechanisms that can be invoked to account for a larger animal's apparently greater resistance to oncogenesis (on a unit weight per unit time basis). For example, if "spontaneous" neoplasms are in reality caused by environmental chemical carcinogens, and these carcinogens are available to both the larger and smaller animals in dose packages of equal size, the dilution factor in the larger animals would be relatively large and the carcinogenic threshhold level would rarely be reached. The tremendous fat depots in whales might sequester fat-soluble carcinogens and thereby prevent exposure of other tissues. Another possibility, however, is that in the processes of evolution, defense or resistance mechanisms are developed on what might be thought of as a balanced construction basis. That is, genetic and epigenetic mechanisms are integrated in the animal's constitution in such a way that breakthrough of aberrant populations is possible only at a level consistent with survival and propagation of the species. This might be called the one-hoss shay concept, deriving from the philosophy in Oliver Wendell Holmes' poem, "The Deacon's Masterpiece," (*72*) :

> Now, in building of chaises, I tell you what,
> There is always somewhere a weakest spot,—
> In hub, tire, felloe, in spring or thill,
> In panel, or crossbar, or floor, or sill,
> In screw, bolt, thoroughbrace,—lurking still,
> Find it somewhere you must and will,—
> Above or below, or within or without,—
> And that's the reason, beyond a doubt,
> That a chaise breaks down, but doesn't wear out.

The concept of "balanced construction" to insure against breakdown of weak components carries implications in relation to simple versus complex animals and in relation to the currently *apparent* rarity of neoplasms in the invertebrates. For example, a hydra might be looked upon as a complete organism of about the same size and degree of complexity, in terms of different cell types, as a single mammalian hair follicle (admittedly, this is a gross underestimation of hydra, but serves for argument). A defense or resistance mechanism in hydra must be adequate to assure survival and reproduction of only one such unit. On the contrary, a dog with several millions of hairs must defend against aberrant cells in *any one* of these millions of units, for a breakthrough in any one of these units can lead to death of the entire organism just as readily as a single breakthrough in a hydra. We do not know the exact incidence of hair follicle neoplasms in dogs, but we are certain it is less than one per several hundred dogs. This being the case, and assuming that the breakthrough rate for hydra as individuals is roughly that for individual hair follicles, imagine the number of hydras one might have to examine to find a single neoplasm. This number would have to equal the complete hair populations of at least several hundreds of dogs—an astronomical number. Add to this the fact that one is greatly aided in the detection of hair follicle tumors by a magnification factor inherent in the gross size that tumors can reach in dogs, as opposed to a very limited size attainable in a hydra, and we find little difficulty in understanding why anomalous growths in hydra have so rarely come to the investigator's eye.

Carrying this analogy (at some risk) to nonbiological systems, one might think of the number of fail-safe mechanisms that engineers must build into a modern jet aircraft, as opposed to those required to prevent failure of a vastly more simple flying machine such as a trainer of 1940 vintage. It is evidence that the available engineering skill (equivalent in this analogy to the genetic information stored in every somatic cell) must be deployed far more extensively and efficiently to provide protection at the whole-machine level for the machine with system components several thousand-fold more numerous than those of the "primitive" machine. This analogy if carried further could become all too terrifying and should be tempered with the reminder that the amount of genetic information contained in a cell nucleus of an organism such as hydra is not remarkably less than that in the nucleus of a human cell. The engineering talent (informational input) available in the design of a modern jet aircraft, on the other hand, is vastly greater than that used in the design of the primitive trainer; therefore, proportionately more information can be applied to fail-safe features of the contemporary model.

Is it possible that hydra has deployed its informational content toward perfecting its limited number of systems to the point where aberrant population breakthroughs would be infinitesimally rare, whereas the "higher" metazoans have sacrificed this advantage for utilization of information toward multiplication and diversification of systems? This is in essence the question that Good answers in the affirmative, pointing to cellular immune mechanisms as the superimposed fail-safe mechanism provided to compensate for increased systems at risk. Good believes all is lost when the fail-safe systems fails. This is acceptable reasoning, but it breaks down in view of the cell-population-at-risk consideration presented above, *unless* the basic unit at risk is a particular specialized cell group, such as the aggregate of liver cells, rather than the individual liver cell itself. This is the crux of the problem. For if the unit at risk is one liver or two lungs, etc., rather than 10^9 liver cells as opposed to 10^{15} liver cells (mouse vs. whale), then the whale is at no greater risk than the mouse or any other animal with an equal number of systems and an equally efficient surveillance (fail-safe) mechanism.

But where is the mechanism that makes 10^9 cells no more prone to aberrations than 10^{15} cells? Perhaps we see it at work in the phenomenon of limited *in vitro* lifetime of human diploid cells, first described by Hayflick and Moorhead (*73*). Their experiments, later confirmed by others, showed that normal human embryonic lung fibroblasts could give rise to only approximately 50 cell generations. After that, the cells entered a phase of crisis and then died. In rare exceptions, estimated on the order of $<1\%$ (*74*), human diploid cells undergo alterations including karyotypic changes and then are able to proliferate indefinitely. Does this suggest that each animal has a built-in program insuring against replication error in each cell type, commensurate with organ size and cell replacement requirements, and relating to patterns of lifespan and predisposition to neoplasia? Experiments to answer this question could perhaps be done by comparing cells from mammals at the extremes of size range and lifespan. However, another opportunity would be afforded by determining generation capacities of cells from invertebrates such as planaria and hydra that can reproduce asexually *ad infinitum*. The importance of doing studies on generation capacity of invertebrate cells in tissue culture seems very evident, yet because of technical difficulties progress in this field has been slow (*75*). While it is known that some insect cells can proliferate indefinitely *in vitro* (*76*), it is not known whether these cells have neoplastic potentialities when they are returned to hosts of the species of origin.

In this Monograph, Hadorn's work (*18*) seems to supply some insight into the problems posed above. In his experiments, the imaginal discs of *Drosophila* may be equated with the organ or system units that were postulated above as the units at risk of developing aberrant populations. In contrast to the findings of Hayflick working with mammalian cells, Hadorn found that the embryonic cells of the imaginal discs can go on reproducing themselves through more than 300 generations without losing the ability to differentiate into normal organ units when returned to larvae that undergo metamorphosis. In other words, larval *Drosophila* cells appear to be "immortal."

This could be interpreted as evidence overthrowing the concept of allotment of a limited number of cell generations to particular cell types. But it could equally well be interpreted to show a fundamental difference between mammalian and insect cells. Do we have here evidence supporting the idea that animals lacking the thymic system have other intrinsic safeguards operating effectively to prevent the appearance of aberrant cell populations? The exceptions seen by Hadorn, in which atypical organs eventually appeared or the transplants (atelotypic) failed to differentiate under the influence of host metamorphosis, support the expectation that some failures of intrinsic control should occur. These exceptions, as Gateff and Schneiderman suggested (*19*), seem to represent examples of the neoplasms so difficult to find among invertebrates. The importance of Hadorn's and of Gateff and Schneiderman's studies when considered in relation to Hayflick's can hardly be overestimated. But differences in methods and the cell sources necessarily used in these works warn against attaching too much significance to the differences found.

Relation of Sensitivity to Ionizing Radiation and Susceptibility to Neoplastic Change

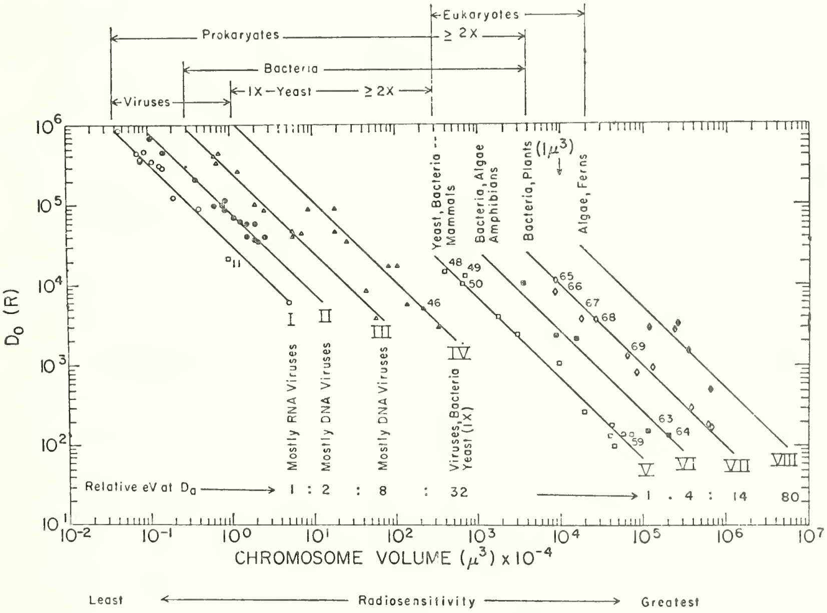
Data recorded less than 20 years ago (*77*) indicated that warm-blooded vertebrates have an LD50 in a relatively narrow range below 1000 r for total-body irradiation. Cold-blooded vertebrates such as *Triton* sometimes have an LD50 as high as 3000 r, while certain mollusks have LD50's as high as 8000–20,000 r, amebae 100,000 r, and ciliates as high as 350,000 r. These figures might suggest that animals of the lower phyla have some kind of inherent resistance to lethal effects of ionizing radiations, and if so, these animals might also have resistance to the carcinogenic effects of ionizing radiation.

However, tremendous irregularities in sensitivity to total-body irradiation exist, as Bacq and Alexander noted (*78*). The apparent resistance of cold-blooded vertebrates can probably be accounted for in part by their lower body temperature. In frogs, radiation damage at a given dose level increases with temperature. But pigeons, which have a higher temperature than mammals, are 5 times as resistant as rats. An actinid coelenterate, *Anemonia sulcata*, is not detectably injured by 100,000 r (*78*), but brown hydras are severely damaged (tentacular degeneration) by 9600 r (*79*). This difference is apparently accounted for by a greater sensitivity of hydras to peroxides produced in water under oxygenated conditions. Unicellular organisms are said to be generally resistant to high levels of radiation, but the fungus *Phycomyces blakesleeanus* shows some sensitivity to 0.001 r (*80*), while *Micrococcus radiodurans* has a D_0 at 70,000 rads (*81*). Most adult insects are resistant to high levels (64,000 r for adult *Drosophilia*), and the adult parasitic wasp *Habrobracon* even has an increased lifespan after receiving 180,000 r (*82*).

Different mechanisms of lethal action of radiation can account for some of these apparent paradoxes (*e.g.*, sensitivity to peroxides of some animals, as already noted), but differences in the response systems of particular animals are probably more often the deciding factors. It is a general principle that rapidly proliferating cells are more sensitive to irradiation than slowly proliferating or nonproliferating cells; this can account in part for the resistance of adult insects, in many of which there is little cell proliferation except in the sex cells, where sterilization occurs at dose levels below the organismal LD50. Bacq and Alexander (*78*) argue that more than the proliferation factor is involved in the radioresistance of insects, since cysteamine-protected rats are permanently sterilized by 700 r, whereas *Drosophila* require 64,000 r before they are sterilized. They suggest that the high levels of amino acids, which maintain osmotic pressure in the body fluids of insects, protect against radiation damage. They further suggest that "it would be interesting to know the radiation resistance of the octopus since its has a gland containing hydroxytryptamine which was found on a weight basis to be the most effective protective agent known."

Despite these rationales, the proliferating embryos of *Drosophila* have an LD50 of only 200 r at 3 hours of age, 500 r at 4 hours, and 810 r at 7½ hours. (*78*). These data indicate sensitivities not markedly different from those of mammals whose deaths are attributable to damage to vital organs in which high proliferative rates prevail (blood-forming organs, skin, gastrointestinal epithelium). Information of this sort gives rise to serious doubts that, on a stoichiometric basis of initial DNA vulnerability, any gradient of radiosensitivity exists as one moves distally along the branching tree of phylogeny. Sparrow *et al.* (*83*) have shown that generally (with some outstanding exceptions) cellular radiosensitivity correlates with the total chromosomal volume in the irradiated cell. However, this correlation held true only if their data were plotted along regression lines, of which 8 could be resolved (text-fig. 4). This led Sparrow and co-workers to conceive of sorting organisms according to "radiotaxa." This is certainly a fresh concept, but one that is likely to be disturbing to classical taxonomists, as it places in a single taxon, for example, such diverse organisms as algae and amphibians. Furthermore, as Sparrow and co-workers observed, organisms such as *Bacillus megatherium* phage α and

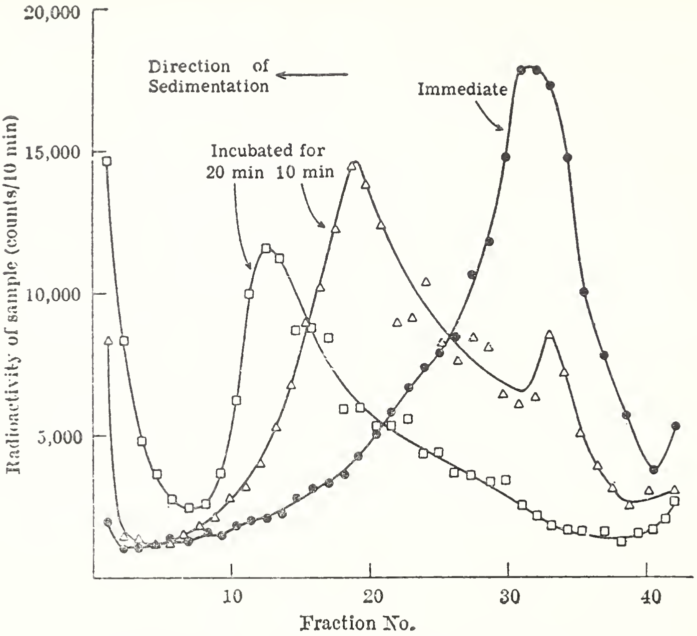
Escherichia coli B_{II} have chromosome volumes differing by a factor of 10^4, but have nearly equal radiosensitivities. Such comparative data are valuable, for they compel a search for the mechanisms that must provide explanations for radiotaxa. One wonders, for example, if the correlation between sensitivity to cyanide and to ionizing radiation, as discussed by Bacq and Alexander (*78*), could be relevant to the radiotaxonomic groupings. It seems rather likely that variable capabilities to repair X-ray damage will have some bearing on how these groupings come to exist. However, it has recently been shown (*81*) that mammalian cells, like the much more resistant *M. radiodurans*, have an efficient system for rejoining radiation-broken DNA strands (text-fig. 5). This process may be passive, according to studies of Elkind *et al.* (*84*). An adequate explanation for differences in radiosensitivity does not, therefore, seem to be in immediate prospect. Possibly relevant to the question is evidence recently reported (*85*) that a photo-dynamic mechanism of repair of DNA after ultraviolet radiation damage is available to certain elasmobranchs, but not to mammals.



Correlation between D_0 and chromosome volume. Points fitted to slopes = −1. Chromosome volumes were multiplied by 10^4 to facilitate computing data. Arrow indicates position at which chromosome volume equals one cubic micron ($1\ \mu^3$).

TEXT-FIGURE 4.—Organisms arranged according to "radiotaxa."

Reproduced with permission of authors and publishers, from Sparrow, A. H., Underbrink, A. G., and Sparrow, R. C.: Chromosomes and cellular radiosensitivity. I. The relationship of D_0 to chromosome volume and complexity in seventy-nine different organisms. *Radiat Res* 32: 915–945, 1967.



Changes in the sedimentation profile of the DNA from *L5178Y* lymphoblasts during incubation after they have been irradiated with 30 krads under nitrogen.

TEXT-FIGURE 5.—Repair of radiation-induced breaks in DNA strands from mouse leukemia cells. Peaks at 10- and 20-minute intervals after irradiation indicate increasing polynucleotide chain lengths with passage of time.

Reproduced with permission of authors and publisher. From Lett, J. T., Caldwell, I., Dean, C. J., and Alexander, P.: Rejoining of X-ray induced breaks in the DNA of leukemia cells. *Nature* (London) 214: 790–792, 1962.

The impact of these important observations in relation to comparative studies of radiation carcinogenesis suggests that they should be taken into account by those who plan and interpret experiments on the carcinogenic effects of ionizing radiations on animals of various phyla. In addition to radiotaxa, will we find that there are also neoplastic taxa, in the sense intimated by Schlumberger (*86*)? And if so, how closely will neoplastic taxa coincide with the radiotaxa? A great deal of work will be required before these questions can be answered, for the data provided by Sparrow *et al.* (*83*) include little information on the invertebrate metazoans. The report by Mix and Sparks (*87*) in this Monograph represents a step in the continuing estimation of the carcinogenicity of ionizing radiation in invertebrates.

Epizootiology of Neoplasia

One of the early hopes of investigators in comparative oncology was that through animal research the causes of human neoplasms would be found. There continues to be such a hope, but it is sobering that not one of the several recognized causes of cancer in man was found through animal observations or experiments. Many chemical, physical, viral, and parasitic agents are known to induce neoplasms in animals, but those that have been found active also in man were known to be so before the animal experiments were done.

Currently, of course, vigorous efforts continue to determine whether certain viruses that induce tumors in animals may also do so in man. The same is true for chemical carcinogens, and possibly the aflatoxins, which have been shown to be carcinogenic in rainbow trout (*88*), may in time be shown to be carcinogenic in man. The nitrosamines are perhaps even better candidates for this distinction, particularly since they have been shown to be carcinogenic in subhuman primates (*89*).

Meanwhile, the effort should not be abandoned, since it is impossible to extrapolate from past negative results. Certainly, if ionizing radiation and 2-naphthylamine had been tested on the proper animals before human beings were exposed to them, their carcinogenic actions would have been detected. Comparative oncologists will feel well rewarded if their findings lead to identification of the cause of even one type of neoplasm in man.

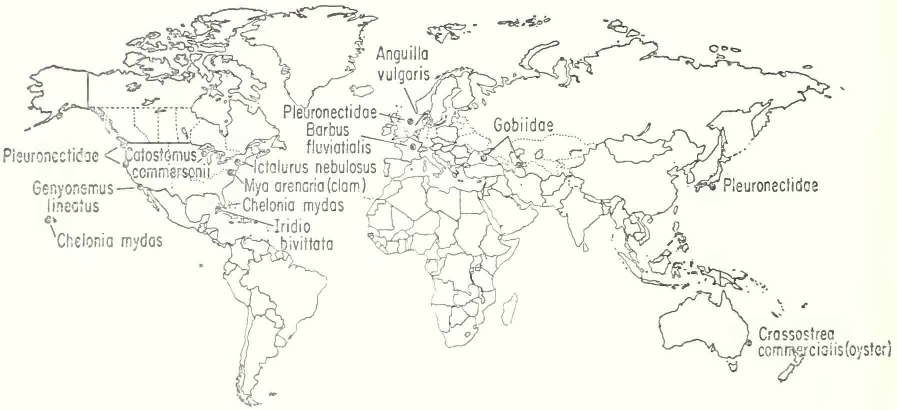
A standard method in cancer epidemiology is to collect incidence data for each histologic type of neoplasm in man and to compare these data among various populations over the world. In this way, cancer incidence relief maps can be made, and it is then possible to try to establish correlations with other "geographic" features, such as climate, industrial activities, ultraviolet radiation exposure, water contaminants (such as arsenic in natural waters), and so on. Of course, the possibilities of establishing valid correlations depend heavily on the accuracy of the data.

In cancer epizootiology the same thing is attempted with neoplasms of animals. This situation is complicated by the fact that many species and many types of neoplasia must be dealt with. Further, there is the double objective of finding correlations between *a*) tumor incidences in a given species and environmental factors and *b*) incidence of tumors in animals and incidence of tumors in man. In *b*), it must be granted that no firm correlations between incidence of animal neoplasms and incidence of human neoplasms have been found.

Nevertheless, a beginning has been made in this field with a few species of animals within the scope of this Symposium. For several years, Wellings and associates have been collecting data on the prevalence of papillomas among pleuronectid fishes in Puget Sound. In this Monograph Cooper and Keller (*90*) report similar studies in San Francisco Bay. They could not discover the cause of these growths, but they did reveal some interesting features concerning age, species predilection, and distribution of animals with the disease. Cooper and Keller found only a single species of flounder, *Parophrys vetulus*, affected in San Francisco Bay, whereas Wellings *et al.* (*91*) found that 4 species may manifest the disease in Puget Sound. The papillomas are found only in fish at ages starting at the early weeks after hatching and going up through the third year. Papilloma-bearing fish are found only in certain portions of San Francisco Bay, which suggests either a relationship to particular genetic pools of the fish—a postulate favored by Cooper—or to the geographic localization of some inducing factor. Chemical and biological agents related to industrial waste or sewage disposal have been considered as possible causes, but none has been strongly implicated.

Papillomatosis of eels, reviewed by Deys (*92*), is another anomaly of growth and form carrying epizootiological interest. During some 25 years, this disease seems to have spread westward along the coasts of the Baltic Sea and then southward along the Danish, German, and Netherlands coasts. If one assumes that the data on which this pattern of spread is based are accurate, the etiology seems more likely to be a viral or biological agent than a chemical one, as there is no corresponding pattern of progression of industrial or sewage pollutions. Text-figure 6 shows the proximity of an area in the North Sea, where papillomas have been found in flounders, to the areas in which eels have been found with papillomas. So far there is no evidence that the papillomas of these different species of teleost fish are in any way related except in their histologic characteristics. An obvious difference in their anatomical sites is that the eel papillomas occur nearly always on the mouth, whereas those of pleuronectids occur anywhere on the body surface. This difference could relate chiefly to the differences in feeding habits and methods between eels and flounders. Eels are mainly scavengers that nose out their food by pushing their heads into crevices or the shells of dead mollusks, whereas flounders generally feed on live fish and crustaceans, though, like eels, they lie close to the bottom.

Lymphosarcoma of northern pike (*Esox lucius*) seems to be an epizootic disease in the lakes and streams of Ireland. Mulcahy (*93–95*) reported an



TEXT-FIGURE 6.—Geographic locations in which papillary cutaneous or pallial growths have been found in the aquatic animals named. *See* Wellings, this Monograph, for a more complete tabulation of papillomas in fish. *Pleuronectidae* = flounders and sole; *Genyonemus lineatus* = white croaker; *Catostomus commersonii* = white sucker; *Ictalurus nebulosus* = brown bullhead; *Anguilla vulgaris* = Atlantic eel; *Barbus fluviatilis* = barbel; *Iridio bivittata* = slippery dick; *Gobiidae* = gobies, gudgeon; *Chelonia mydas* = green sea turtle; *Mya arenaria* = soft shell clam; *Crassostrea commercialis* = rock oyster.

overall prevalence of lymphosarcoma in pike in Ireland of about 1 per 800 fish, in a total sample of over 300,000. An apparently similar condition was observed among muskellunge (*E. masquinongy*) in Lake Scugog of Ontario, Canada (*96*). The etiology is unknown for this condition in either species, and it follows that no etiologic relationship is known between this condition and hematopoietic neoplasms of other animals.

Only recently, Wolf (*8*) discovered papillary epitheliomas of the mantle in rock oysters (*Crassostrea commercialis*) of 2 widely separated estuaries on the east coast of Australia. These growths were uniform in their histologic features, and although only a few were found among two large populations, the possibility of some environmental factor as their cause must be considered. The same is true for the lesions resembling hematopoietic neoplasms of *C. virginica* and *C. gigas* reported by Couch (*6*) and Farley (*7*).

Codreanu (*97*) gave an interesting account of a disease in mayfly larvae he considers similar to leukemia. Leukemia-like proliferations of hemocytes were described in the hemoceles of mayfly larvae parasitized by the larvae of another insect, of the chironomid family. Whether or not the disease in question is closely analogous to leukemia is not clear, but the stimulation of hemocyte proliferation by a parasite is certainly a phenomenon worth investigation. Further, the parasitization was accompanied by transfer of a microsporidan from the chironomid to the mayfly. This additional bit of information is especially interesting in view of the ability of certain microsporidans to stimulate cell hypertrophy and sometimes proliferation as well [*see* Sprague (*98*), this Monograph].

In text-figure 6 are mapped the locations of some of the papillomatous diseases of several species of teleost fish, 1 species of marine turtle and 2 species of bivalve mollusks. This map includes only the more common examples involving fish; Wellings' review (*25*) has a more complete listing. No etiologic relationship is implied by placing these diverse species on a single map. The only feature common to these lesions is their papillary character and origin from integument. It is notable that papillomas of pleuronectids have been seen in fish off the European shores of the Atlantic Ocean as well as off the Japanese and North American coasts of the Pacific Ocean.

In text-figure 6, papillomas are indicated in the green sea turtle, *Chelonia mydas*, off the Florida Keys as well as off the Hawaiian Islands. The histologic features of the lesions from the various geographic sources appear to be different, judging from a limited number of specimens submitted to the Registry of Tumors in Lower Animals by Waddell (*99*) (Florida Keys) and Smith (*100*) (Hawaiian Islands). The lesions from Florida turtles have mixed fibrous and epithelial components, whereas those from Hawaii are more nearly pure fibromas of the dermis, with polypoid gross features.

The etiology of any of the papillomas of marine and freshwater aquatic animals is unknown. Only the papillomas of catfish (*101*) have been shown to be transplantable, but as an experimental system, these papillomas have not been explored nearly to the extent that papillomas of rabbits and other mammals have. Papillomas on the mouth of the white croaker, *Genyonemus lineatus*, though apparently quite common in this fish in certain localities and absent in others (*102*), have not been investigated as intensively as they should be.

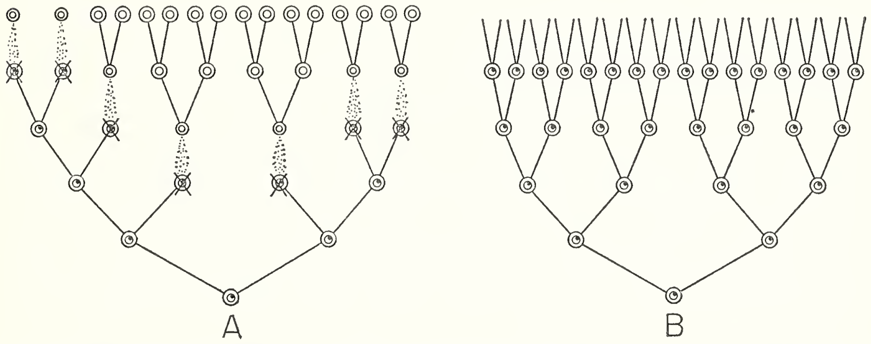
The number of mammalian species in which virus-induced papillomas occur is quite large, and knowledge of evolutionary relationships among the papilloma viruses might be advanced if it is found that viruses are of etiologic importance in many of the fish papillomas. The association of virus particles with hyperplastic epidermal lesions of walleyed pike, described by Walker (*103*) in this Monograph, is a recent finding of special comparative interest because the virus is morphologically rather similar to the mammary tumor virus of mice.

Definition, Characterization, and Phylogenetic Origin of Neoplasia

To this point, I have been discussing neoplasia as if there existed a definition of it. But, as yet, there is no *constitutive* definition of neoplasia, or of even a single neoplasm. The "mal-structure of triplet" in Strehler's verse (*1*) is a concept still to be proved or disproved, and it is unlikely that even the most optimistic molecular oncologist expects to find any two neoplasms for which *the* "mal-structure of triplet" is identical, even though certain constitutive features may prove to be shared by many neoplasms. In the meantime we have had to settle for working, or operational definitions, with which textbooks abound. While these have pragmatic value to students and teachers, the damage they do by discouraging development of new concepts may be one of the greatest impediments to cancer research.

For example, many definitions include the phrase, "uncontrolled growth," which certainly requires some qualifications with regard to many tumors, *e.g.*, some prostatic cancers and mammary cancers that respond sharply to endocrine control factors, sometimes for years. "Uncontrolled" therefore comes to mean more or less controlled as compared with certain other proliferative processes, and depending on physiological conditions existing in the host. Even the property of serving no useful function to the host is not without exception as, for example, in a functioning thyroid neoplasm that restores the euthyroid condition to a host previously in a hypothyroid state. Besides, the "no useful function" character does nothing to distinguish neoplasms from hypofunctioning or hyperfunctioning conditions involving no threat to the host on a cell-proliferative basis. Data on cell kinetics in normal tissues and in neoplasms reveal that tumor cell populations may proliferate at rates higher than, lower than, or equal to those of their normal counterparts, and that the lifespan of proliferating tumor cells and size of the nonproliferating pool of cells in neoplasms can vary widely and overlap the normal ranges (*104–109*) Cell population kinetics following transplantation of experimental tumors can vary in the most fundamental ways imaginable, and yet these variations may not be detected by the observer who depends on the eye and the microscope unaided by special tools of analysis.

For example, in text-figure *7B* are diagrammed the cellular derivations following transplantation of mouse leukemia cells into a secondary, histocompatible F_1 hybrid host. In text-figure *7A* are diagrammed the cellular derivations following transplantation of a virus-induced fowl sarcoma, as analyzed by Pontén (*110*), using sex chromosomes to mark the donor cells. In the first case (*7B*), a new tumor arises solely from proliferation of the donor cells, as demonstrated by transplantability of the new tumor back to a mouse of the parent strain—a result that could not be obtained if the tumor in the F_1 host had arisen from the F_1 host's cells rather than from the original donor's cells. In the second case, however (text-fig. *7A*), the tumor that emerges in the new host is composed first of donor cells, later of mixtures of donor cells and virus-transformed host cells, and eventually of virus-transformed host cells alone. Thus without any ostensible discontinuity in its growth, the cell population constituting the "transplanted" tumor has changed from donor origin to host origin.



Text-figure 7.—Differences in cell derivations in transfer of a fowl sarcoma (*A*) and a mouse leukemia (*B*) to secondary hosts. Sex-chromosome marker is represented by *black dot on margin* at nucleus. *A:* Donor cell lineage becomes extinct, and the new tumor, induced by virus in the presence of donor cells, is composed of transformed cells of the recipient. Virus is represented by *small dots*. *B:* Tumor in the new host is composed of grafted cells derived from the original donor.

These arguments and examples are presented to encourage new workers in the field of comparative oncology—particularly new investigators of anomalies of growth and form among invertebrates—to retain flexible and skeptical attitudes toward prevailing concepts of the nature of neoplasia. Much of the terminology developed and used for expediency by clinical cancer workers, and even by investigators of experimental tumors in the higher animals, can have subtle but far-reaching influences on conceptual thinking. These influences may be serious "hang-ups" which the investigator of neoplasia in lower animals is in an enviable position to avoid.

Most mischievous is the tradition of categorizing neoplasms as "benign" and "malignant." There is no better way short of direct experience to combat this temptation than to become acquainted with the writings of Foulds (*111*), from whom the following quotation is taken:

> Many independently variable unit characters enter into the complex phenomenon of "malignancy." "Malignancy" is essentially a clinical concept; it is invaluable in clinical practice and is useful for colloquial reference to a lethal mode of behaviour, but discussion and investigation of "malignancy" as though it were an indivisible entity or unitary quality of tumour cells are meaningless and futile. The associations of unit characters that contribute to malignant behaviour are liable to wide variation and the characters need separate specification and study. It is at least arguable that the fundamental processes of neoplasia may be revealed most clearly in benign tumours which represent neoplasia in its simplest form, "without the frills" and uncomplicated by the numerous additional characters that complicate the study of malignant tumours. Some of these additional features may be of great interest and convenience to laboratory investigators without being essential to the neoplastic process or at all important in determining the course and outcome of neoplastic disease in animals or in man.

Foulds' six principles of tumor progression and his precepts of "unit character" definition of particular neoplasms (*111*) will be recognized immediately by biologists as an attempt to place tumor development and character expression in a framework formed of the fundamental units of Mendelian genetics and the processes of developmental biology, embodying the same elementary processes with which evolutionary science is concerned. [Recall Alfred Wallace's use of the term "progression" to mean the continual departure of new animal species from preceding types (*112*)]. It is certain that the future will see the Fouldsian principles defined more precisely in the language of molecular biology. A diagrammatic representation of how a neoplasm may manifest the phenomenon of progression has been borrowed from Hauschka's dissertation (*46*) (text-fig. 8). As Foulds states (*111*): "The recognition and analysis of tumor progression depend on the study of identifiable characters that can be *separately* specified and described and measured." This has been done with excellence in studies by Potter (*113*) of the specific proteins produced by experimentally induced plasma cell neoplasms of mice. Potter found that, among morphologically indistinguishable plasma cell neoplasms, each produced an electrophoretically distinguishable globulin. In Potter's experience, this "unit character" remained constant (after initial variation and selection processes were passed through) for a given neoplasm transplanted serially, under uniform conditions, through many generations of the inbred strain (BALB/c) of mouse of origin.

The opportunities offered by *Drosophila* for analysis of unit characters of neoplasms are obvious, in view of the great amount of genetic information available for this insect. The occurrence of polyteny in dipteran salivary glands adds to the usefulness of *Drosophila*, for, if neoplasms of salivary gland can be obtained, it will be possible to correlate sites of chromosomal puffing, representing sites of gene activation, with the unit characters manifested by respective tumors. With the transplantation

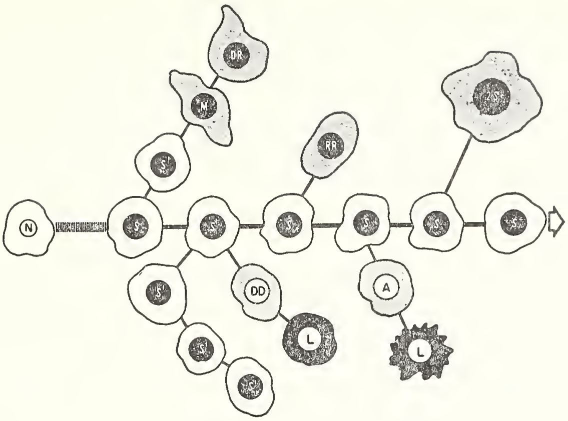


Diagram illustrating tumor progression through stem-line variability. The nuclear symbols represent the following cell types: *N*, nonmalignant cell; *S*, main stem-line; S^1, secondary stem-lines; *2S*, polyploid stem-cell variant; *M*, metastatic cell; *DR*, drug-resistant mutant; *RR*, cell with increased radiation-resistance; *DD*, drug-dependent mutant; *A*, antigenic mutant; *L*, mutations lethal for individual tumor cells.

TEXT-FIGURE 8.—Phenomenon of progression in neoplastic cell lineages. Acquisition or loss of unit characters gives rise to new "species" represented by branches from the stemline.

Reproduced with permission of author and publisher. From Hauschka, T. S.: The chromosomes in ontogeny and oncogeny. *Cancer Res* 21: 957–974, 1961.

methods reported in this Monograph by Hadorn (*18*) and Gateff and Schneiderman (*19*), the study of salivary gland tumors becomes a distinct possibility in the near future. However, chromosomal puffs are not the only mark by which gene activation can be recognized. Gel electrophoresis, as used by Barber and Steward (*114*) in studying sequential activation of specific enzyme syntheses in developing tulips, could be used in a similar way to determine activation of product synthesis in insect tumors, if one assumes that sufficient tumor tissue can be obtained by transplantation methods.

These lofty thoughts of elegant experiments need to be supplemented by some down-to-earth rules of thumb. One of the most common questions asked when an investigator not experienced with neoplasms discovers an anomaly of growth and form is: How do I tell whether or not this is a neoplasm, and if it is, how should I go about classifying it?

The best answer to this question is to suggest that the proper procedure is similar to that followed when one is required to identify an unfamiliar or a previously unknown organism or species. A neoplasm may be thought of as a community or kinship of cells that occupies a certain niche within the host ecosystem, has certain habits of growth, movement, and reproduction as well as certain nutritional and metabolic requirements, puts into its environment certain products and excreta, and has certain morphologic properties at levels ranging from gross through ultrastructural to molecular. A neoplasm also passes through developmental stages, responds to particular stimuli and noxious agents, and can be parasitized or infected. The cells composing these communities or kinships have variable and perhaps greater tendencies to differ from one another than cells of "normal" tissue kinships, and during progression (in the Fouldsian sense) manifest a process combining features of evolutionary radiation on the one hand and embryogenesis on the other (*i.e.*, combining genetic and epigenetic changes). They endanger the continuation of the host ecosystem, and the manner and speed with which an individual neoplasm does so are important among its characteristics. In the aggregate, neoplasms, like species, can be grouped according to major common characteristics, but as individuals none is exactly like any other.

Taxonomic keys can be developed for classifying neoplasms, but none has ever been widely accepted—partly because for a single species such as man it has not proved clinically profitable to characterize a neoplasm beyond the organ and cell type of origin, histologic pattern, extent of host involvement, and the likelihood and rapidity with which it destroys the host. Many classifications of neoplasms exist, of course, and as with classifications of animals, these continually change. Questions about some diseases have been debated for decades as to their neoplastic nature or classification, revealing that inadequate definitions, inadequate data, or both, underlie the difficulty.

Hope has often been expressed that, as the causes of various neoplasms are found, classifications can be revised around an etiological framework. This can be done, as it has been, for example, with infectious diseases, but it will probably always be necessary for oncologists to use classifications based on the biologic and morphologic characteristics of the neoplasm-host interaction, just as pathologists have characterized and classified infectious diseases according to the host response, *e.g.*, pyogenic versus granulomatous infections.

At present, it is far more important that biologists characterize the neoplastic diseases or related disorders of growth and form they observe than that they apply "correct" names. To encourage this, a tabulation of parameters is provided below. It is not complete, and to experienced oncologists it may appear elementary and sophomorish; but it requires only a little familiarity with the literature to appreciate that case reports of the past would be much more useful if the descriptive portions had been more complete. At the same time, no first encounter with a possibly neoplastic disease should be withheld from publication simply because circumstances may not allow evaluation of all the parameters listed here.

I. Macroanatomical Characteristics

1. Anatomic location
2. Size
3. Shape, surface properties
4. Color
5. Consistency
6. Boundary features
7. Necrosis
8. Ulceration
9. Invasion, gross
10. Metastasis, gross

II. Microanatomical Characteristics

1. Tissue and cell type of origin
2. Stromal characteristics and vascular supply
3. Degree and kind of histologic and cytologic atypicality
4. Unicentricity versus multicentricity
5. Invasiveness
    (*a*) *In situ?*
    (*b*) Local invasion—fat, fascia, bone, etc.
    (*c*) Lymphatic invasion
    (*d*) Blood vascular invasion (or hemocele)
    (*e*) Perineural invasion
    (*f*) Other
6. Metastases—*cf.* primary
7. Cytochemical characteristics
    (*a*) Pigments
    (*b*) Enzymes
    (*c*) Intracellular storage (glycogen, mucin, keratin, hormones, fats, etc.)
8. Extracellular products (collagen, reticulin, cartilage, bone, amyloid, shell, chitin, etc.)
9. Karyotype
    (*a*) Modal number and distribution pattern of chromosomes
    (*b*) Qualitativeabnormalities (breaks, deletions, additions, translocations, fusions, marker chromosomes, etc.)
10. Ultrastructure
    (*a*) Changes of organelles, endoplasmic reticulum: mitochondria, plasma and nuclear membs.; lysosomes, Golgi, tonofibrils, myofibrils, microtubules, desmosomes, etc.
    (*b*) Infectious agents
        Viruses, bacteria, fungi, parasites
11. In tissue or cell culture
    (*a*) Cell morphology (fibroblastoid, epithelioid, ameboid)
    (*b*) Structure-forming capability (papillae, glands, fascicles, etc.)
    (*c*) Cell adhesiveness
    (*d*) Cell cohesiveness
    (*e*) Contact inhibition of movement
    (*f*) Contact inhibition of division
    (*g*) Pinocytotic activity
    (*h*) Membrane properties (zeiosis, microfilopodia, etc.)
    (*i*) Nuclear properties (pulsation, rotation, amitosis, etc.)

III. Macrobiological Characteristics

1. Growth continuous or intermittent?
2. Growth reversible?
3. Lethality
4. Responsiveness (hormones)
5. Dependency (hormones)
6. Growth rate
7. Growth in ascites form
8. Transplantability
9. Progression
10. Radiosensitivity
11. Drug sensitivity
12. Host age, sex developmental stage
13. Functional activities (pigment changes, endocrine target effects)

IV. Microbiological Characteristics

1. *In vivo*
    (*a*) Relation to host's genomic constitution (hereditary control)
    (*b*) Transmissibility by infectious agent
    (*c*) Antigenic profile (histocompatibility antigens, fetal antigens, virus-specific antigens, paraproteins, etc.)
    (*d*) Metabolic properties (functional products such as protein and steroid hormones, serotonin, histamine, etc.)
    (*e*) Response to antimetabolites, hormones
    (*f*) Cell kinetics (lifespan, mitotic time, intermitotic time, size of nonproliferating pool, etc.)
2. *In vitro*
    (*a*) Nutritional requirements
    (*b*) Growth rates
    (*c*) Cloning efficiency
    (*d*) Gain or loss of antigens
    (*e*) Gain or loss of transplantability
    (*f*) Inducible enzymes, *e.g.*, transaminases
    (*g*) Other properties as studied *in vivo*

LOWER ANIMAL NEOPLASMS

Neoplastic "Equivalents"

Although neoplasms are generally conceived of as disorders afflicting individual metazoans and higher plants, "neoplastic equivalents" have been hypothesized to exist or to be possible at higher orders as well as at lower orders of life organization. The best known analogy at a higher level of organization is one that places man in the role of the cancer cell population and the entire earth's ecosystem in the role of the host that may be destroyed through a combination of man's proliferation and his disposition to upset the steady-state condition of the host. Thought-provoking as this analogy may be, it is of more use to sociologists and ecologists than to oncologists, because it does not yield specific information on the structure of neoplasia at or below the cellular level.

Neoplastic equivalents have been conceived at levels below metazoan organisms for many years. Bashford (*24*) in his report of 1904 inserted the following footnote:

> These characters mark the malignant new growths as new organisms, morphologically and physiologically equivalent to the parent one which they are invading. The one-tissue (homogeneous) character of the malignant parenchyma makes them resemble a protozoan colony, the offspring of one conjugation rather [than] a metazoan embryo or adult. The invasion of one malignant growth by another finds its protozoon parallel in the struggle for existence between two slightly dissimilar protozoon colonies which are the offspring of distinct conjugations.

Clearly Bashford was thinking of the evolution of new and dominating forms of protozoa as a process comparable to the appearance of neoplastic cells in a metazoan ecosystem. After Bashford's time, Metcalf (*115*), Mottram (*116*), and Ord (*117*) described protozoa which they likened to cancer cells, mainly on grounds of nuclear and morphologic anomalies. Spencer and Melroy (*118*) developed similar concepts in experiments with bacteria (*Eberthella typhosa*), in which mutants evolved in response to repeated exposure to temperature conditions incompatible with life of the starting bacterial populations. They suggested "a possible connection between the adaptive process in bacteria and the carcinogenic process in mammals." Gause (*119*) reviewed the subject of neoplastic equivalents among single-celled organisms. He believed neoplastic equivalence could be defined in terms of Warburg's hypothesis (*120*), a hypothesis that has not been universally accepted, though it has been supported defiantly in some quarters (*121*).

In 1952, Sonneborn (*122*) drew a comparison between neoplastic cells and populations of paramecia having the κ factor. This factor enables paramecia to kill off other paramecia not possesssing the factor, resulting in a shift from a mixed population system to a monotypic population, under the artificial conditions of the experiments.

van Wagtendonk (*123*), in this Monograph, reviews the knowledge currently available on the κ, λ, μ, and other Greek-lettered particles related to paramecium metabolism and ecology. The presence of λ in a population of paramecium relieves these organisms of their dependency on folic acid for continued multiplication and survival. Such λ-bearing paramecia thus become, in a sense, equivalent to some types of neoplastic cells, *e.g.*, mouse mammary tumors (*124*), that have progressed from a state of dependency on estrogen to one of independency relative to that hormone requirement. The λ-bearing paramecium may also be compared with murine virus-dependent leukemias, in which available evidence has failed to disprove that continued presence of the leukemia virus is necessary for the leukemia cells to proliferate. (This RNA virus-tumor system differs from some DNA virus-tumor systems in which recoverable virus may apparently be lost from the tumors.)

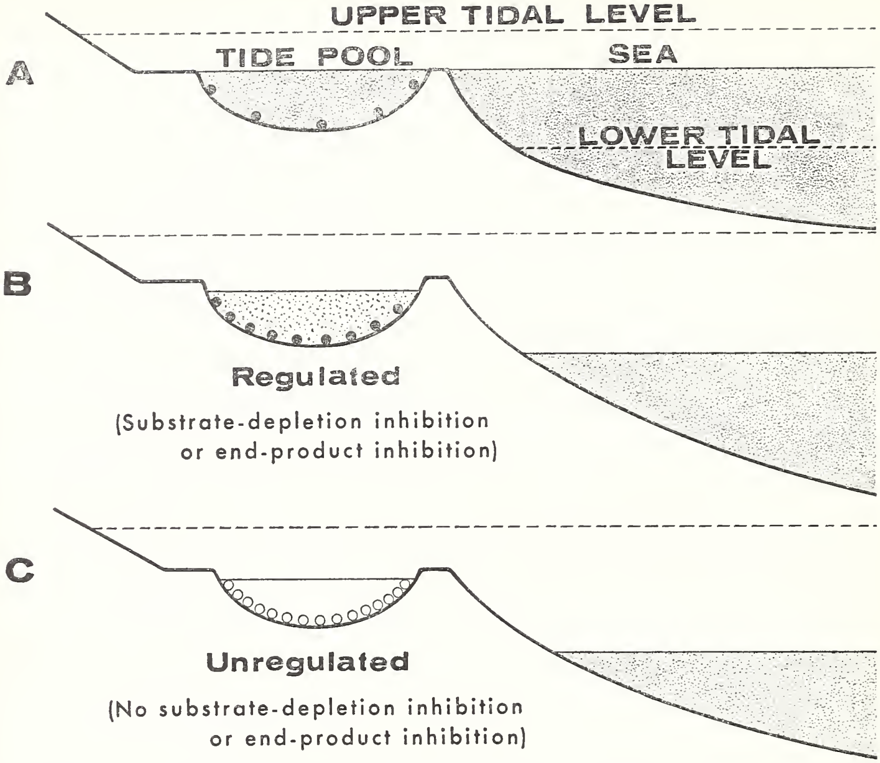
However, van Wagtendonk chooses to designate the λ particle itself, not the λ-bearing paramecium, as a neoplastic equivalent. In other words, neoplasia in this instance comes in a package, which can be put into the cell or taken out of it. This concept is disturbing, since most oncologists, while amenable to the thought of taking the cancerous nature out of the cell, find it hard to conceive of taking the cell out of its central role in cancer, which is what van Wagtendonk seems to propose in his hypothesis. The thought is nevertheless consistent with the molecular biologist's definition of cancer as "malfunction of triplet," *i.e.*, with moving the definition of neoplasia below the cellular level.

While neoplasia in metazoans is bound, because of the cellular composition of metazoans, to manifest itself through cell activities, it is possible to construct a model of neoplasia as it might have existed in evolutionary times before cells as we know them today had evolved. In doing so, one is forced to suggest that a neoplastic equivalent may have been the first form of "pre-life."

The first self-replicating organic macromolecules (pre-life forms) presumably originated in the ancient seas or "primaeval broth" (*125–127*). Existing in a volume of substrate that was, for practical purposes, infinite, these self-replicating macromolecules or coacervates should have had no restraints on their rates of replication other than those of chemical reactivities and mass action laws. A fixed and preferably rapid rate of replication relative to the rate of degradation of these pre-life forms would have been conducive to their continued existence and increase.

However, when such fixed-rate replicating forms found themselves caught up in tide pools, the situation would have been changed. A fixed rate of replication then could have led to exhaustion of substrate before the next high tide replenished the substrate and diluted the population (text-fig. 9). If the degration rate of such forms were high enough, none would have survived until the next tide. Thus might have been created the conditions favoring selection of the first biochemical systems of growth regulation. In this hypothetical situation, the fixed-rate replicating pre-life form would be equivalent to neoplasia, in the sense that absence of any growth-regulating mechanism in the tide-pool ecosystem would lead to the destruction of the entire ecosystem.

LOWER ANIMAL NEOPLASMS


UPPER TIDAL LEVEL
A
TIDE POOL
SEA
LOWER TIDAL LEVEL
B
Regulated
(Substrate-depletion inhibition or end-product inhibition)
C
Unregulated
(No substrate-depletion inhibition or end-product inhibition)

TEXT-FIGURE 9.—The survival advantages of pre-life forms with growth-regulatory mechanisms over those lacking such mechanisms. *Fine stippling* represents substrates in "primaeval broth." *Large solid black spots* represent a replicable pre-life form. *A:* Tide pool has just been filled and the pre-life form begins to assimilate substrate and to replicate. *B:* Tide has fallen and begun to rise again. The pre-life form with feedback inhibition mechanisms has multiplied, but has slowed its generation time, so that the substrate is not exhausted and will permit continued replication until the incoming tide replenishes substrate. *C:* Pre-life form lacking feedback inhibition mechanisms has completely exhausted the substrate and all individuals of the pre-life form have undergone degradation (*open circles*). Through exhaustion of the ecosystem, this form has not survived in the tide-pool niche. The pre-life form in *B* is analogous to "normal," regulated cells, that in *C* is analogous to neoplastic, unregulated cells. The tide pool with its substrates is analogous to metazoan host.

If, by "accident," some mechanisms of growth regulation based on feedback effects of substrate depletion or end products occurred in one of the pre-life forms, this form would have a selective advantage in the tide-pool situation. By slowing its growth rate in response to substrate depletion or end product accumulation, such a modified form could survive to the next tide, when rapid replication could be resumed until substrate again decreased in concentration. Furthermore, such a regulable form would be in a position to compete successfully against fixed-rate replicators having either higher or lower rates of replication. Such a pre-life form, with the capability of adjusting growth to environment, might be thought of as the first ancestral form of organisms adapted to limited and finite ecosystems. It would correspond to the modern equivalent of "normal" cells, which respond to environmental conditions in a way that is advantageous to the perpetuation of the ecosystem and of the organism within that system. In a disconcerting way, the development of neoplastic cell populations in contemporary metazoans resembles not only a reversal of evolutionary processes but also a recapitulation of them. In transforming from a more regulated condition to a less regulated one, the cancer cell seems to represent evidence that the law of primacy of survival of the individual at the cell level has not been forgotten and can at times supersede the law of survival at the organismal level. But in metazoans the law of organismal survival can be superseded by the law of cellular survival only at rates that do not jeopardize continuation of a given metazoan as a species. Any organism so constituted as to allow too frequent and early occurrence of neoplasia within its community of cells would become extinct. Thus we are brought again to look for the mechanisms that protect against aberrant cell populations, whether these mechanisms are built into the system design of organism development through "genetic stability" (possibly via repair mechanisms) or whether they are made up of special fail-safe systems (immunologic mechanisms).

These reflections may be disparaged because they seem not to suggest any practical approach toward control of neoplasms, and in fact seem to say that a certain amount of neoplasia is inevitable, as the result of life forces that are as old as life itself. The latter may be true, but these thoughts should have heuristic value. By retracing the steps followed in the phylogenetic development of regulatory mechanisms, are we not likely to find understanding of these mechanisms, just as developmental biologists expect to reach such understanding by retracing ontogenetic development? Regrettably, it does not necessarily follow that with understanding will come control of neoplasia. An escape from this uncomfortable thought lies in the advice of Michael Faraday: "But try anyway; no man knows what is possible."

> When the stars threw down their spears,
> And water'd heaven with their tears,
> Did He smile his work to see?
> Did He who made the lamb make thee?
> (From William Blake's "The Tiger")

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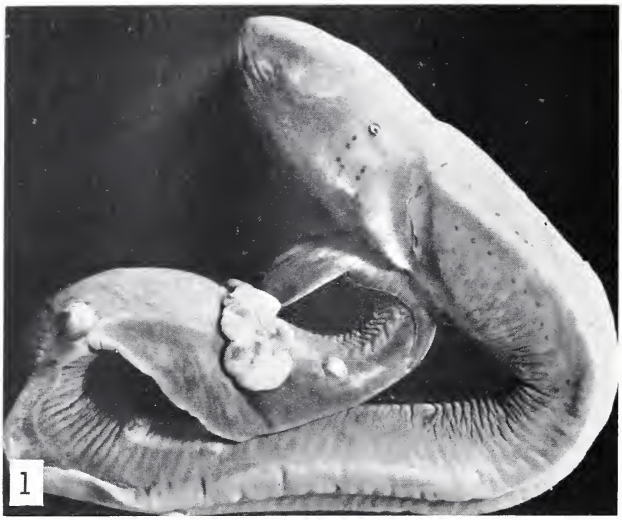

FIGURE 1.—Adult sea lamprey (*Petromyzon marinus*), bearing multiple cutaneous and subcutaneous tumors. Five tumors are visible in this dorsal view of the animal in convoluted posture. Accession No. 89, Registry of Tumors in Lower Animals, The Smithsonian Institution, Washington, D.C. Submitted by J. H. Howell, Bureau of Commercial Fisheries, Hammond Bay Biological Station, R.F.D., Millersburg, Michigan. Reduced 2 ×

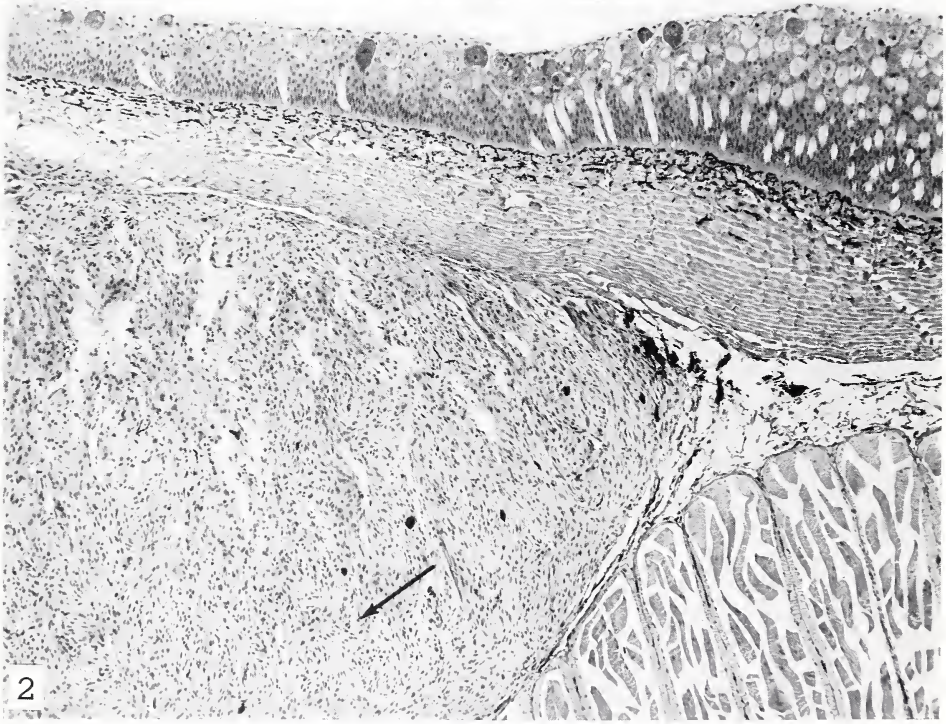

FIGURE 2.—Histologic section of one of the neoplasms from specimen in figure 1. *At right center*, the edge of the neoplasm interposes itself between skin (*above*) and skeletal muscle (*lower right*). The tumor is sharply circumscribed and is composed of fibrocytes disposed in swirling bundles. In some areas (*arrow*), nuclei tend toward a double-column arrangement characteristic of Verocay bodies in neurilemomas. Melanocytes (*black spots*) are within the tumor and at its margin *on right*. Hematoxylin and eosin. × 60

Essential Relationship Between the Lymphoid System, Immunity, and Malignancy[1]

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SUMMARY

Malignancy and immunity can be considered to be antithetical adaptive processes. Successful malignant adaptation reflects in some way defective immunologic adaptation. A primary *raison d'être* for the lymphoid system and certain immunities is surveillance against the malignant adaptation. From this perspective the high frequency of malignancy in patients with immunologic deficiency and in patients under immunosuppression is to be expected. The relations of chemical carcinogens to immunosuppressives and the experimental association of all manner of immunosuppression with development of primary and induced malignancy seem also to be entirely congruous. It is postulated that the essential interplay between the lymphoid system, immunity, and malignancy can be understood as a developmental phenomenon, in which immunity and malignancy are adaptations derived from a more fundamental, highly advantageous impulse to variability that appeared phylogenetically among forms ancestral to the most primitive fishes. If this be so, invertebrates should have, by mammalian standards, poorly developed or no adaptive immune responses and little or no cancer.—Nat Cancer Inst Monogr 31: 41–58, 1969.

IN DISCUSSING tumors of invertebrates, we shall deal with an issue more fundamental than the questions of etiology, pathology, or pathogenesis of any particular tumor. We shall try to argue that an essential relationship exists between immunity, the lymphoid system, and malignancy.

Today there is much excitement among immunobiologists concerning the subject of tumor immunity and the nature of the relationhsip between the host and its malignant tumor. For example, it has become clear that malignancies occur with inordinate frequency when the immunologic apparatus is inappropriately developed either genetically or because of environment. Recently it has been observed that patients receiving effective immunosuppression develop malignancy far too frequently to be explained

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by chance alone (*1*, *2*). Immunosuppressive agents in experimental animals foster the development of malignancies induced by chemical carcinogens and by oncogenic viruses (*3–5*). In addition, apparently all carcinogenic chemicals, when appropriately given to the host, are also immunosuppressive (*6–9*). Where properly studied, it has been found that both virus-induced and carcinogen-induced malignancies of mammals regularly possess antigens foreign to the host which the host does not eliminate effectively (*10–12*).

We will attempt to argue that all of these fascinating relationships are not those of chance, but rather reflect an essential and fundamental relationship between the lymphoid system, immunity, and malignancy which may help us understand why we have not to date been able to identify and define malignant processes in invertebrates.

From our data (*13–22*) and from our review of observations of others (*23*, *24*) we believe that the lymphoid system developed in the lowest vertebrates concomitant with development of adaptive immunity in its earliest form. We believe further that capacity for malignant development, though not necessarily an absolute concomitant, appeared in close temporal association with development of the lymphoid system and adaptive immunologic capacity. The essence of our thesis is that a successful (from the point of view of tumor) malignant adaptation indicates that, for one reason or another, immunologic surveillance against malignant cells has failed. The failure of the host to recognize and eliminate cells possessing foreign antigens at their surface is to us an essential component of the malignant adaptation.

A corollary to this proposition is that potentially malignant cells arise often during the life of all vertebrates but that the defense against these cells is almost always equal to the task and is a function of the forces of immunologic adaptation.

Our recent extension of this view is offered as a challenge to this conference. We believe that malignancies in invertebrates lacking an immunologic system represent an essential incongruity; we propose further that the difficulties, to date and in the future, in finding or producing true malignant processes in invertebrates are a function of this incongruity. We contend that true malignant adaptations do not occur among most invertebrates and, if they do occur, they occur so seldom as to preclude significant selective influence. However, we also contend that malignant adaptations occur with much greater frequency among all vertebrates and that constant presence of malignant adaptation has exerted selective pressure in development of the lymphoid system and immunologic capacity. Even among the primitive vertebrates, malignancies, when found, should reflect *all* the most rapidly replicating cellular systems.

If we are correct, malignancies identifiable by the human oncologist or mammalian oncologist should be found among all vertebrates but should not be found among the invertebrates. If a reasonable series of convincing malignant adaptations among the invertebrates is not demonstrated at this

Symposium, or if the evidence presented indicates that such malignancies only rarely occur in invertebrates, the work of the Symposium will support our view. If, on the other hand, malignancies are found to occur frequently among invertebrates and in a variety comparable to that seen among the vertebrates, the conference will have rejected our hypothesis, and new and perhaps more productive reflections can guide our future work. Let us look at the argument.

Our interest in an essential relationship between immunologic processes and the malignant adaptation began when we examined immunologic processes in Hodgkin's disease which, at the time of our first study, was considered to be a lymphoma. Schier *et al.* (*25*) observed that the disease is often associated with anergy. Indeed, when tested with a battery of six skin test antigens that induce frequent delayed allergic reactions in normal persons, an unusually great number of patients with Hodgkin's disease did not respond. With Dr. William Kelly and several other associates (*26–29*), we confirmed the findings of Schier *et al.* and showed that patients with Hodgkin's disease also failed to reject skin homografts normally or to respond to sensitizing antigens with the usual development of delayed allergy. These patients also resisted transfer of delayed allergic reactions by lymphoid cells from donors having a high degree of delayed allergy. The immunologic deficiency in patients with Hodgkin's disease was similar to that associated with advanced malignancies of other types (*30*); but it occurred earlier in Hodgkin's disease and seemed to be more regular and complete than in most other forms of malignancy (*28*). Even patients with stage-1 Hodgkin's disease are sometimes anergic. Nonetheless, the frequency of anergy clearly progresses with advancing disease (*28*, *31*). Subsequently Lukes and Butler (*32*) showed that progression of Hodgkin's disease in any stage of its development and expression is a function, at least in part, of the percentage of lymphocytes in the involved lesions. Since then, many investigators have confirmed and extended our observations on Hodgkin's disease (*32–36*). Furthermore, the usefulness of Lukes' classification, so interesting to the immunologist, has been established. It would appear to us that Hodgkin's disease is not a lymphoma at all but rather a maligancy involving the stromal reticular cells of the lymphoid tissues. If the immunologically based lymphoid-immunologic reaction is vigorous and the disease is localized to one set of nodes, cure can be obtained by excision or by vigorous local radiotherapy (*37–39*). If the immunologically based lymphoid-immunologic reaction has been compromised sufficiently and the Hodgkin's disease is primarily reticular or fibrotic, recurrence and progression occur even after extensive radiation. The immunologic deficiency in Hodgkin's disease involves primarily the thymus-dependent lymphoid apparatus responsible for the cell-mediated or cellular immunities, of which delayed allergy is a prototype.

LOWER ANIMAL NEOPLASMS

Patients with advancing forms of other malignancies have similar immunologic deficiencies. These patients have been shown to reject foreign tumor cells less vigorously than do normal persons (*30*).

Just as Lukes showed for Hodgkin's disease, Black and others have related prognosis in other malignancies, such as mammary, gastric, and colonic cancer, to the host reaction in the form of invading lymphoid cells (*40–50*). By contrast, patients with Hodgkin's disease did not seem to have much deficit in their humoral immune mechanisms. Plasma cells were increased in the lesions and in hematopoietic tissues, immunoglobulin concentrations were normal, and antibody was produced vigorously (*26*).

Immunologic analyses have also been done on patients with other malignancies of the lymphoreticular system. For example, in multiple myeloma big deficits in production of normal immunoglobulins and antibodies were found, whereas cell-mediated immunity remained relatively intact (*51–56*). In chronic lymphatic leukemia, abnormalities of both cell-mediated immunities and humoral immunities were demonstrated early in the disease (*57*). As lymphatic leukemia progressed, hypogammaglobulinemia occurred and a severe immunoglobulin deficiency was demonstrated in approximately one-half the cases (*58–62*). Although acute lymphatic leukemia and acute myeloid leukemia require more definitive study, presently available observations indicate that immunologic mechanisms are quite well preserved in patients with these diseases until late in the course of the diseases when the immunosuppressive influence of the antileukemic agents takes its toll (*63–67*).

In the framework of our new concepts of the functional organization of the lymphoid system (*5*, *62*), it seems clear that a process intimately involved with pathogenesis of Hodgkin's disease compromises the integrity of the thymus-dependent population of lymphocytes while it leaves relatively intact the function of cells of the thymus-independent system. On the other hand, either the oncogenic process or some direct consequence of this process in patients with myeloma compromises function of the plasma cell system which in turn subserves synthesis of immunoglobulins and circulating antibodies. This process leaves relatively intact the thymus-dependent system of cells responsible for the cell-mediated immunities. In patients with chronic lymphatic leukemia, both systems of cells have severe malfunctions. These findings might be interpreted in terms of the interference with normal differentiation by deviation of lymphoid stem cells at the site of the malignant differentiation; however, other interpretations are possible (*69*, *70*). For example, in Hodgkin's disease, the agent (be it virus or otherwise) inducing the malignant differentiation of the reticulum cells might, like measles or rubella virus, be able to compromise the usual functions and adaptations of the thymus-dependent system of lymphocytes.

In many experimental malignancies of the lymphoid system, agents inducing malignant adaptation have been shown to influence oncogenically the central lymphoid organs where normally differentiation of lymphocyte populations occurs. For example, in 1944, McEndy, Boon, and Furth

(*71*) showed that neonatal thymectomy prevents leukemia in AKR mice. The subsequent work of Kaplan (*72*) and Siegler and Rich (*73*) presented a rationale for this observation. Neonatal thymectomy prevents development of this form of leukemia by removing the site at which the malignant differentiation occurs, just as neonatal thymectomy removes the site for normal differentiation of the thymus-dependent lymphoid system.

In extensive studies with the RPL12 virus-induced lymphoma in chickens, Peterson *et al.* (*66*), Cooper *et al.* (*74*), and Dent *et al.* (*75*) similarly showed that removing the bursa of Fabricius early in life removes the site of development of this lymphoma. Similarly bursectomy in the newly hatched chicken (*76*) or in chick embryos in late stages (*77–79*) removes the site for normal differentiation of the thymus-independent lymphocytes and plasma cells, which ultimately subserve the function of antibody and immunoglobulin synthesis. Consequently, in experimental animals we can define thymus-dependent and bursal (thymus-independent) forms of lymphoid malignancy as a function of the differentiative site in which the malignant differentiation must take place.

Malignancy and the Immunologic-Deficiency Diseases

As patients with immunologic-deficiency diseases have been studied through the years, it has become more apparent that the primary immunologic-deficiency diseases of man are largely associated with malignancy. For example, patients with Bruton-type, sex-linked recessive agammaglobulinemia have developed lymphatic leukemia more frequently than can be explained by chance (*80*).

Lymphatic leukemia appearing in the thymus has been the only malignancy observed in these patients. One can reason that this group of agammaglobulinemic patients, like mice, develop leukemia within the thymus and that this malignancy occurs in the one lymphoid system available. Patients with sex-linked, recessive agammaglobulinemia lack a lymphocyte-plasma cell system responsible for antibody synthesis. It is not yet clear whether their increased susceptibility to lymphatic leukemia beginning in the thymus simply reflects susceptibility to infection with an oncogenic virus or whether it more directly reflects the deficiency of a relationship of humoral immunity and defense against hematopoietic malignancy. It is of interest, however, that Gorer (*81*) in 1958 showed that circulating antibodies afford significant resistance to malignancies of the leukemia type whereas they play a much smaller role in resistance to certain solid tumors. Patients with this form of agammaglobulinemia develop cellular immunities with normal vigor; it is provocative that they have not revealed, thus far at least, a propensity to develop solid tumors such as reticulum cell sarcomas, carcinomas, and other sarcomas, which plague patients with more broadly based immunologic deficiencies. Patients suffering from the late-occurring immunologic deficiencies of varying onset times and expression (*82*) seem to have a much greater tendency to develop malignancy than patients with Bruton-type agammaglobulinemia. For example, these patients have developed lymphomas, reticulum cell sarcomas, carcinomas, and other forms of malignancy with a frequency far in excess of that observed in the general population of comparable age (*5*, *83–85*). Here the relationship of the malignant adaptation to faulty immunologic adaptation seems more clear. Likewise, patients with the ataxia-telangiectasia syndrome, who have a progressive immunologic deficiency in both components of the immune system, develop malignancy with far greater frequency than members of the general population (*5*, *86*, *87*). Lymphoreticular malignancies have occurred in approximately 10% of children with this syndrome who have been observed and studied to date. The immunologic deficiency in patients with ataxia-telangiectasia involves faulty development of the thymus, deficits of cell-mediated immunities, and deficiencies of antibody synthesis and production of the immunoglobulin IgA and IgE (*88*,*89*).

Patients with the Wiskott-Aldrich syndrome have still another form of immunologic deficiency associated with frequent malignant adaptation (*90–93*). This immunologic deficiency appears to reside on the afferent limb of immune response and involves both cell-mediated and humoral immunologic responses (*94–98*). In the lymphoid tissues there is progressive depletion of the lymphocyte populations of the thymus-dependent deep cortical regions. For the most part, malignancies in these patients have been lymphoreticular and have shown a propensity to involve the central nervous system.

Still another clinical disease, in which defective ability to resist infection is associated with a high risk of malignant adaptation, is the Chediak-Higashi anomaly (*99–102*). Patients with this disease have skin and bowel infections with inordinate frequency early in life. Their skin pigment becomes diluted due to concentration of melanin in very large melanosomes in the pigment-containing cells of the dermis (*102*, *103*). Indeed, in these patients many of the single membrane-bound organelles are abnormally large, which has been considered to be due in some way to a genetically determined anomaly in lysosomal formation (*103*, *104*). Thus far, the defect of bodily defense responsible for susceptibility to infection has not been defined. Clearly, however, if children with this anomaly live long enough, they develop a pleomorphic invasive disease of the lymphoreticular apparatus. Invasion of peripheral nerves as well as the central nervous system is a regular concomitant (*105–107*). Whether or not this is truly a malignant disease has been argued; however, once this disease develops, its progression is malignant, and cells from the lymphoreticular tissues clearly behave in many ways like malignant cells (*107*). Of particular interest is the fact that when malignancy occurs in these patients many hematologic elements contain virus particles (*108*).

The final lesson to be derived from these clinical associations is that each of the many different forms of immunologic deficiency, when studied sufficiently long, has been associated with an inordinate susceptibility to malignant adaptation.

The possible bases of this association are numerous. One could reason that the malignancies are but additional evidence of susceptibility to infectious oncogenic agents. One can also reason that the excessive stimulation of the lymphoreticular apparatus from exogenous antigens, resulting in greater proliferative activity of cells directly or indirectly involved in immune responses, has predisposed these populations to malignant change. However, the simplest relationship, and consequently the one most likely to be correct, is that the genetically determined immunologic deficiencies in these patients have compromised the vigor of a basic surveillance function of the lymphoid system and have tipped a delicate balance in favor of a constant pressure to malignant adaptation.

Aging, Immunologic Involution, and Malignancy

Recently, experimental studies have clearly shown that immunologic vigor flags in association with involution of the lymphoid tissue at higher ages (*109–112*); a programmed involution of a thymus and thymus-dependent lymphoid system, very probably under endocrine control, can very well be postulated. For example, in some strains of mice, the involution of the lymphoid system and demonstrable fall of immunologic vigor can be shown to occur as inverse functions of a propensity to expression of autoimmune phenomena and development of apparently spontaneous malignancy (*113–116*).

If immunologic aging is, indeed, linked with a propensity to successful malignant adaptation, under certain circumstances as it seems to be, these findings show still another intimate link between immune responsiveness and malignancy.

Association Between Effective Immunosuppression and Malignancy

Chemical and immunologic suppression of immunity in experimental animals favors transplantation of malignant tumors, development of metastases, and the occurrence of both virus-induced and chemical-induced malignancy (*3–5*).

By interfering with development of the thymus-dependent lymphoid system and cell-mediated and homograft immunities, neonatal thymectomy likewise fosters the development of certain chemical-induced and virus-induced malignant tumors (*117–122*). In these relationships one sees again a reciprocal relationship between an effective immunologic adaptation and a successful malignant adaptation. Some of these relationships hold also for man. A by-product of organ transplantation in man has been recent evidence that immunosuppression, sufficient to prolong survival of allogeneic organs within the human body, will permit homotransplantation of malignant tumors (*123–129*) and will permit occurrence of primary malignancies with astonishing frequency (*130–135*). In the early days of organ transplants from cadavers, kidneys were sometimes transplanted from patients dying of malignant disease. In some 9 patients, not only were the allogeneic kidneys successfully transplanted, but also the malignancy was successfully transplanted. In at least 3 of these patients, the transplanted malignancy became widely disseminated, only to be completely rejected, presumably by an immunologic mechanism, upon discontinuation or reduction of immunosuppressive treatment. These observations focus on the effectiveness of the immunologic mechanism as a defense against transplantation of cancer. They also reveal the potential vigor of an immunologic process in achieving elimination even of large amounts of solid tumor tissue if the immunologic process can be effectively brought to bear on the malignancy as in these transplanted tumors.

Perhaps even more provocative have been the numerous recent observations of development of primary tumors in patients under immunosuppressive therapy. During the last year or two, approximately a dozen such cases have been observed. The occurrence of a malignant adaptation under the influence of immunosuppressive therapy deserves intensive and critical study which is not yet available, but at this writing the incidence of malignancy in patients subjected to immunosuppression seems disproportionate to the incidence of malignancy in immunologically normal persons of the same age. Whereas some of these tumors have been lymphoreticular, others have been epithelial and still others anaplastic. It seems that effective immunosuppression in man, particularly when such immunosuppression involves potent antilymphocytotoxic serum, permits or fosters the malignant adaptation.

Immunologic Surveillance

All of the relationships between immunity, the lymphoid system, and cancer cited above are essential derivatives of a hypothesis originally stated by Thomas (*136*), subsequently promulgated effectively by Burnet (*137*, *138*), and investigated experimentally by Klein (*139*, *140*). The lymphoid system, especially but not exclusively that component associated with cell-mediated immunities, has been viewed as an efficient police system capable of discerning foreignness and eliminating cells deviant in surface antigenicity from host cells. Malignant tumors are known to possess antigenicity foreign to the host. When this foreignness encounters a lymphoid system in appropriate balance, elimination of the "foreign" cells results.

Genetic or exogenously induced deficiencies of this surveillance function should favor malignant adaptation. Where this hypothesis has been tested to date, it has proved correct in every instance.

Balance of Adaptations

Both immunologic reaction and the occurrence of malignancy can be viewed as biologic adaptations. Thus one sees in the interaction of these potential adaptations a remarkable interplay. The immunologic adaptation of mammals has evolved over several hundred million years as an efficient means of ultimately eliminating even the last of foreign invaders. To complete a successful immunologic adaptation, a congeries of immune reactions can be called upon, including cell-mediated responses, antibodies of a variety of molecular forms, and several extraordinary amplification systems, *e.g.*, the complement system, the angry or activated macrophages, phagocytes of other varieties, poorly understood kinins, and inflammatory and coagulation resources. To mention only a few possible resources, malignant adaptation, on the other hand, may involve: immunologic tolerance (*120*, *141–145*), immunologic enhancement (*146*), sneak-through mechanisms, associated surface antigens widely spaced on cells or of "weak" nature, growth potential of tumor, possible control of delivery of effective host cellular response (*147*), antigenic modulation (*148*), or even anti-inflammatory principals.

The evidence that a dynamic interplay exists between these two adaptions substantially supports the essentiality of their relationship. From this perspective, it seems to us no coincidence that immunity and malignancy so often present extraordinary interfaces and interrelationships. It seems no accident that: 1) All chemical carcinogens are immunosuppressive; 2) all immunosuppressives foster carcinogenesis and oncogenesis, even though they are not carcinogenic in themselves; 3) experimental procedures, such as graft-versus-host reaction and neonatal thymectomy that depress immunologic vigor, foster the development of malignancy; 4) effective immunosuppression in patients is accompanied by a rapid rise in malignancy; 5) diseases of immunologic deficiency are associated with too frequent malignancy; 6) malignant diseases are often accompanied by demonstrable immunologic deficiencies; 7) the incubation period of certain malignancies is accompanied by demonstrable defects in immunologic function; 8) advanced age is a period of declining immunologic vigor when cancer frequently develops; and 9) once malignancy has occurred, second primary malignancies are of great frequency.

These findings seem necessary corollaries of the basic concept that the primary *raison d'être* for immunologic adaptation is to provide defense against malignant adaptation.

Essential Relationship Between Malignancy, the Lymphoid System, and Immunity

Studies in phylogenetic perspective, though incomplete, suggest that the fundamental relationship between immunity, the lymphoid system, and cancer is to be better understood only when one realizes that capacity for both of these adaptations evolved together and that both evolved as a function of a more basic internal impulse to variation which itself had imposing survival advantage.

While studying the development of immunity, we discovered that the lymphoid system and adaptive immunity, as we know them in man, appeared among forms ancestral to the most primitive fishes. This, of course, does not mean that the roots and rootlets of adaptive immunity are not to be found in invertebrates. Certainly they will be, but to date we and others have been unable to define the full range of adaptive immune reactions among invertebrates. Lymphocytes defined by features that characterize mammalian lymphocytes appeared for the first time phylogenetically among the most primitive of fishes (*149–152*). Seemingly congruous with this sequence is the knowledge that malignant tumors are frequently found in fishes, amphibians, reptiles, birds, and mammals and that they are strikingly similar in all levels of vertebrates. Thus, as mammalian oncologists we can easily distinguish the malignancies of reptiles, amphibia, and all manner of fishes including the lamprey. In our own work, for example, we have found invasive malignancies of lamprey, *e.g.*, invasive mesotheliomas (*152*) and neurilemomas (*153*). By contrast, we have not yet been satisfied that the lumps and bumps or cellular accumulations among the invertebrates have features that are associated with the malignant tumors of mammals.

Carcinogenic chemicals have produced malignant tumors in all vertebrate forms, but none have yet produced malignancy in either hagfishes or invertebrates (*154*, *155*).

A final parallel deserves comment. All vertebrates, whether man, mouse, bird, reptile, amphibian, marine or freshwater teleost, holostean, chondrostean, or lamprey, have circulating round cells which are readily destroyed by radiation, nitrogen mustard, or adrenal steroids. Invertebrates do not seem to have such cells (*150*). Further, all vertebrates are killed by total-body irradiation in a dose of about 1000 rads (*150*). Yet invertebrates, even potentially growing forms, resist lethality in doses one or two logs greater than this (*150*, *151*).

Though still meager, these observations suggest a hypothesis which can explain all of the strange relationships outlined in this summary. We suggest that the capacity for malignant adaptation as well as for immunologic adaptation developed from a more fundamental change. For example, it could be that great survival advantage was derived from capacity for variation at the level of the cell nucleus. A change in control of such variation might be based on relatively simple alteration of nuclear homeostasis, *e.g.*, loss of a nuclear repair enzyme or alteration of the enzymatic basis of nuclear response to ionizing radiation. Once it had appeared, a change favoring an impulse to variation in differentiation, somatic mutation, or even in genetic material could have been of major survival advantage and would, therefore, have been perpetuated in all forms. One consequence of such a major step may have been capacity for frequent malignant adaptation; another may have been capacity for immunologic adaptation. The immunologic adaptation, in turn, could have developed progressively a capacity for surveillance against the threat of the malignant adaptation, and the malignant adaptation (together with programmed involution of the immunologic system) may have had survival advantage in protecting the ecologic niches against exhaustion by individuals of each developing vertebrate form.

Our position is highly speculative. Its corollaries, however, present numerous working hypotheses which can be disproved and are thus of scientific merit (*156*). One of the most proximal of these corollaries is that malignancies either do not occur or occur only rarely among the invertebrates. It is with this hypothesis that we would like to challenge this Symposium. A delightful achievement of this conference would be to prove us wrong and to establish that invertebrates do, indeed, have malignancies which occur with the frequency and variety so readily observed among all the vertebrates.

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Neoplasia and Primitive Vertebrate Phylogeny: Echinoderms, Prevertebrates, and Fishes—A Review[1]

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SUMMARY

This review detected no definite examples of neoplasia among the Echinodermata, Hemichordata, Urochordata, Cyclostomata, and Holocephali. There is one instance of a neoplasm in a cephalochordate: a chromaffinoma in *Branchiostoma*. In the cartilaginous fishes, eight different histogenetic types of neoplasia were reviewed, of which melanomas were the most frequent. Among the bony fishes, mostly types characteristic of mammals were encountered. The commonest of these were neoplasms of skin, fibrous tissue, and pigment cells. One can conclude that the tumors of fishes are similar in structure and physiology to their counterparts in higher vertebrates. There is reason to believe that the capacity to form neoplasms under appropriate environmental circumstances is an intrinsic property of living systems.—Nat Cancer Inst Monogr 31: 59–128, 1969.

THIS REVIEW covers most of the modern literature dealing with neoplasia in echinoderms, prevertebrates, and fishes. In addition, some hyperplastic lesions and reversible or self-limiting reactions to injury are included, because these either resemble neoplasia or are the only descriptions of cellular proliferative reactions available in the group concerned.

It is recognized that a biological definition of neoplasia is not generally applicable for the selection of material for review, because the biological potential of most tumors in these animals remains unknown. It is therefore necessary, except in certain well-known instances, to base diagnostic judgments largely on the classical morphological criteria of the histo-

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[1] The International Pacific Halibut Commission is gratefully acknowledged for providing some of the specimens illustrated, including the codfish with pharyngeal tumors. The fibroma of the Coho salmon presented is the property of Dr. R. E. Millemann of the Department of Fisheries and Wildlife, Oregon State University. The literature search and preparation of the manuscript would have been impossible without the help of Mrs. Beverly Cartwright and Miss Virginia Jentoft. Several of my colleagues and good friends have contributed in various ways to the studies of skin tumors in pleuronectids summarized in the text. These include: Dr. Laurence M. Ashley, Dr. Harold Berkson, Dr. Robert E. Brooks, Mr. George Brown, Dr. R. G. Chuinard, Dr. Robert A. Cooper, Dr. Robert L. Fernald, Dr. Richard T. Gourley, Dr. John E. Halver, Miss Virginia Jentoft, Dr. Eugene Kozloff, Dr. Glynn McArn, Dr. Bruce S. Miller, Mrs. Mary Rau, Dr. Albert K. Sparks, and Mr. Cleve Vandersluys.

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pathologist. Historically derived from studies of mammalian tissues, these criteria include increased mitotic rate, anaplasia, invasion, and metastasis. Their general validity among lower vertebrates and invertebrates is not yet firmly established.

Moreover, it was usually necessary, particularly in the instance of older references, to accept the descriptions and diagnostic judgments of others without the opportunity to examine the crucial material. In some cases, diagnoses and species names are reworded in the interest of uniformity, or in what seems to be more modern terms. The words "neoplasm" and "tumor" are used interchangeably in the text that follows.

Some of the older work, which has been reviewed (Schlumberger and Lucké, 1948; Lucké and Schlumberger, 1949; Scharrer and Lockhead, 1950; Nigrelli, 1954; Schlumberger, 1957; Finklestein, 1960), is mentioned only in tabular form. Examples of singular importance to the experimentalist, or to the student of epizootic diseases, are treated in greater depth. A discussion at the end of the manuscript deals with some problems in comparative neoplasia.

TUMOR INCIDENCE BY MAJOR TAXONOMIC GROUPS

The major groups of animals reviewed in this study are listed in table 1. Also indicated are the number of reports located and the number of species involved. Note that there are no reports for a number of broad taxonomic categories.

Table 1.—Major taxonomic groups reviewed*

	Number of literature citations reviewed: Neoplasms or related lesions	Number of species involved
Phylum Echinodermata	2	3
Phylum Chordata		
Subphylum Hemichordata (enteropneustes, tongue worms)	0	0
Subphylum Urochordata (tunicates)	5	2
Subphylum Cephalochordata (lancelets, Branchiostoma)	1	1
Subphylum Vertebrata		
Superclass Pisces		
Class Agnatha	0	0
Subclass Cyclostomata (hagfishes, lampreys)		
Class Chondrichthyes	11	10
Subclass Elasmobranchii (sharks, rays, skates)		
Subclass Holocephali (chimaeras)		
Class Osteichthyes	278	179 + 3 hybrids
Subclass Sarcopterygii (lungfishes, lobefins)		
Subclass Actinopterygii (higher bony fishes)		

*Classification of chordates after Lagler, K. F., Bardach, J. E., and Miller, R. R. *Ichthyology.* New York, John Wiley & Sons, 1962, 545 pp.

ECHINODERMATA

Tchakhotine (1938) reported the experimental production of a lesion he believed to be neoplastic in the blastulae of sea urchins of the species *Paracentrotus lividus*, *Echinus microtuberculatus*, and *Psammechinus miliaris*. The embryos were treated with sodium monobromoacetate and sodium monoiodoacetate, added to the sea water. The mesenchymal cells, which normally migrate into the blastocoele, began to proliferate in an uncontrolled fashion, and in several hours, filled the blastocoele completely, preventing gastrulation. The cells continued to proliferate, displacing the ectodermal elements and finally causing disintegration and death of the embryo. The proliferative activity of the mesenchymal cells continued even when the embryos were returned to fresh sea water lacking the active compounds. The embryos had, therefore, apparently been destroyed by an excessive, irreversible, atypical, unregulated overgrowth of a single cell type.

In another report, Thomas (1931a) had previously described a somewhat similar occurrence when the unfertilized eggs of *P. lividus* were treated with carotene crystals for approximately 24 hours. The eggs were then transferred to fresh sea water without carotene crystals and fertilized. During gastrulation the mesenchyme proliferated in an excessive and uncontrolled manner, filling the interior. The embryos became opaque and motionless, and only rarely assumed a form suggesting the tetrahedral shape of the normal pluteus larva.

One could interpret the above experiments in two ways. 1) The observations reflect a disturbance in embryonic regulation produced by the application of metabolic poisons and are not examples of neoplasia. In this regard, it is well known that a variety of metabolic poisons and mutagenic agents disrupts various aspects of development in numerous species. 2) The observations reflect the development of neoplasia analogous to the embryomas, *e.g.*, nephroblastoma, neuroblastoma, hepatoblastoma, known to occur in the young of higher vertebrates.

It appears that these abnormalities of embryogenesis are at least related to neoplasia, in that they are excessive, unregulated, irreversible (?), more or less massive overgrowths of one cellular component at the expense of others. The relationship of such overgrowths to neoplasia might only be in the operation of similar underlying mechanisms, and a neoplasm might then be viewed as an improbable and usually detrimental developmental manifestation occurring at some time in the life of a plant or animal. The value of such a view is that it implies the vast literature of experimental embryology is pertinent to the study of neoplasia, and that an eventual understanding of development will reveal the basic changes underlying the genesis of neoplasia.

A number of attempts were made to induce neoplasia in postembryonic echinoderms by the injection of carcinogenic hydrocarbons, or other substances, but there are no recorded examples of a successful result. In one such experiment (Kaiser, 1965), carcinogenic hydrocarbons were given by various routes to *Asterias rubens*. It was suggested that the failure to obtain a positive result was related to physiological factors (such as rate of absorption) characteristic of the taxonomic group involved. Table 2 summarizes the available literature mentioned above, together with some unpublished data.

UROCHORDATA

There are no unequivocal reports of neoplasia in urochordates. However, Siedlecki (1901a and 1901b) reported tumor-like hypertrophy and hyperplasia of epithelial and connective tissue cells in the intestine of the ascidian *Ciona intestinalis*, associated with infection by the sporozoan *Monocystis ascidiae*. This intracellular parasite produced massive enlargement of cells, eventually leading to cytolysis. In some cases there was hyperplasia of adjacent epithelial and connective tissue cells, resulting in a tumor-like structure that remained after the disappearance of the parasites. The author compared these tumors to hepatic adenomas in mammals. It is well known that cells proliferate, and/or become hyperplastic, in response to many and various intracellular and extracellular parasites, such as viruses (*e.g.*, lymphocystis disease), microsporidia, myxosporidia, rhizopods, ciliates, trematodes, etc. (Caullery, 1952; Nigrelli, 1959). Although in some instances such reactions may eventually lead to, or border on, neoplasia, Siedlecki's descriptions do not clearly represent such an event.

J. A. Thomas (1931b and 1932) inoculated *Bacterium tumefaciens*, the etiological agent of crown gall disease, into the tunic of *Ascidia mentula*. This maneuver resulted in a zone of necrosis and intense inflammation, but nothing resembling neoplasia. In one individual a conspicuous nodule formed, but this subsequently regressed between the 10th and 15th days of the experiment.

In other experiments, Thomas (1931c) injected arsenic, indol, and coal tar into the tunic of *A. mentula*, resulting in an atypical inflammatory reaction; again, there was nothing resembling neoplasia. The literature relative to urochordates is summarized in table 3.

CEPHALOCHORDATA

There is a single report of a neoplasm in a cephalochordate: a chromaffinoma observed in *Branchiostoma lanceolatum* (amphioxus) (Stolk, 1961). The tumor was irregular in shape, well encapsulated, and apparently originated from the enterochromaffin cells of the midgut. Microscopically, there were large cells in which fine brown granules were observed following fixation in chrome salts. The histopathology of the tumor was similar to that of the same neoplasm in man.

Table 2.—Responses related to neoplasia in echinodermata

Reference	Species	Experimental procedure	Observations
Thomas, J. A., 1931a	*Paracentrotus lividus*	Treatment of eggs with carotene.	Uncontrolled and excessive proliferation of mesenchyme during gastrulation.
Tchakhotine, 1938	*P. lividus, Echinus microtuberculatus, Psammechinus miliaris*	Treatment of embryos with sodium monobromoacetate or sodium monoiodoacetate.	Uncontrolled and excessive proliferation of mesenchyme in the blastula.
Kaiser, 1965; Kaiser and Bartone, 1966	*Asterias rubens, Asterina gibbosa, Solaster papposus, Asterias rubens*	Treatment of postlarval animal with carcinogenic hydrocarbons.	Nothing resembling neoplasia.
Bacon, R. L., unpublished	*Strongylocentrotus purpuratus*	Microinjection of 3-methylcholanthrene into plutei.	Nothing resembling neoplasia.
Wellings, S. R., unpublished	*Pisaster ochraceus, Strongylocentrotus drobachiensis*	Injection of benzo[*a*]pyrene into the coelom, various doses, 20 animals of each species, killed 6 months later.	Nothing resembling neoplasia.

Table 3.—Tissue responses in urochordata

Reference	Species	Experimental procedure	Observations
Siedlecki, 1901a and 1901b	*Ciona intestinalis*	None; spontaneous response to parasitic sporozoan, *Monocystis ascidiae.*	Cellular hypertrophy and hyperplasia; nothing resembling neoplasia.
Thomas, J. A., 1931b and 1932	*Ascidia mentula*	Inoculation of *Bacterium tumefaciens* into tunic.	Necrosis and inflammatory response; nothing resembling neoplasia.
Thomas, J. A., 1931c	*A. mentula*	Inoculation of arsenic, indol, and coal tar into tunic.	Inflammatory response; nothing resembling neoplasia.

CHONDRICHTHYES

There are several reports on neoplasia in elasmobranchs (sharks, rays, skates), as indicated in table 4. Most have been reviewed previously (Schlumberger and Lucké, 1948). Most of the descriptions are of surface tumors; this need not imply that such tumors are necessarily more common, but only that surface tumors are more readily recognized. Interestingly, an unusual number of melanomas occurs among the elasmobranchs. Most of the descriptions indicate that the melanoma cells invaded surrounding tissue, and the tumors were therefore biologically "malignant."

Stolk (1965c) described an invasive epithelioma (epidermoid carcinoma) in an adult female cat shark, *Scylliorhinus catulus.* The tumor was located on the oral mucosa of the lower jaw, behind the lip, and was broadly sessile. Microscopically, there were nests and cords of closely packed polyhedral or columnar epithelial cells, supported in a richly vascular stroma. Some portions were papillary, and there was invasion of surrounding connective tissue, muscle, bone, cartilage, and skin.

OSTEICHTHYES

Many neoplasms have been described in the bony fishes. For the purpose of this review, it is convenient to group the tumors as shown in table 5. Note that the *epithelial tumors* are grouped by the organ or organ system involved; the *mesenchymal tumors* are classified according to the tissue of origin. The number of species represented is also given.

Epithelial Neoplasms in Osteichthyes

A-1. Tumors of the Skin and Buccal Cavity

Epidermal papillomas and epidermoid carcinomas are the commonest epithelial neoplasms observed in fishes (table 6). While many authors described isolated examples, there are a number of reports of skin tumors occurring relatively commonly in fish of a particular species, sometimes in a localized geographic area. Such reports suggest a role of environmental factors and/or biological agents in the genesis of the tumors.

Perhaps the earliest report of this character was that of Keysselitz (1908) who observed epidermal papillomas on the lips of barbels, *Barbus fluviatialis.* The disease apparently was common in fish caught in the Mosel River. The structure of the tumor was typical of epidermal papillomas, consisting of folds of multilayered epithelium supported on a branching fibrovascular stroma. The author described intranuclear inclusion bodies in the epithelial cells.

Fiebiger (1909a) noted multiple warty epidermal papillomas on several climbing perch, *Anabas scandens.* All fish had been kept in the same aquarium for 2–3 years and all were the same age.

Table 4.—Neoplasms in chondrichthyes

Reference	Species	Number of fish	Pathology, comments
Schroeders, 1908	*Prionace glaucus* (sand shark)	1	Multiple adenomas of the liver.
Johnstone, 1911	*Raja clavata* (thornback ray)	1	Melanoma, invasive, right side of head.
Drew, 1912	*Raja macrorhynchus* (ray)	1	Possibly fibrosarcoma.
Drew, 1912	*Raja maculata* (spotted ray)	1	Hemangioma.
Johnstone, 1912	*R. clavata*	1	Melanoma, invasive, dorsum of head.
Johnstone, 1912	*Raja batis* (ray)	1	Melanoma, invasive, dorsal surface of left pectoral fin.
Johnstone, 1913	*R. batis*	1	Melanomas, invasive, multiple.
Cameron and Vincent, 1915	*Squalus suckleyi* (dogfish)	1	Thyroid tumor, possibly carcinoma.
Ladreyt, 1929	*Scyllium catulus* (cat shark)	1	Odontoma.
Takahashi, 1929	*Squalus mitsukurii* (dogfish)	1	Chondroma of third lumbar vertebra.
Haddow and Blake, 1933	*R. clavata*	1	Melanomas, two, not clearly invasive.
Thomas, L., 1933a	*Scylliorhinus canicula*	1	Chondroma, skin of right flank.
Thomas, L., 1933a	*S. canicula*	1	Osteoma, trunk, anterior to dorsal fin.
Stolk, 1956c	*Scylliorhinus catulus* (cat shark)	1	Epithelioma (epidermoid carcinoma), invasive.
Wellings, S. R., unpublished	*Squalus acanthias* (dogfish)	1	Fibroepithelial polyps of lips, multiple, associated with infestation of corium and subcutaneous tissue by nematodes (probably a proliferative reaction to parasites, not a neoplasm).

TABLE 5.—Neoplasms in Osteichthyes

Neoplasms	Number of species represented
A. Epithelial neoplasms	
1. Skin and buccal cavity: epidermal papillomas, epidermoid carcinomas, adenocarcinoma	52 + 1 hybrid
2. Dental tumors: odontoma, ameloblastoma	5
3. Pharynx, parabranchial bodies, gills: adenoma, adenocarcinoma, carcinoma	8
4. Stomach and intestine: adenoma, adenocarcinoma	3
5. Liver: cystadenoma, cholangioma, cholangiocarcinoma, hepatocellular adenoma, hepatocellular carcinoma	8
6. Pancreas: adenocarcinoma	1 + 1 hybrid
7. Kidney: adenocarcinoma	4
8. Urinary bladder: adenocarcinoma	1
9. Ovary: adenocarcinoma, teratoma	3
10. Testis: interstitial cell tumor, seminoma (spermatocytoma)	2
11. Thyroid: hyperplasia, adenoma, adenocarcinoma	28
12. Pituitary: adenoma	4
13. Swim bladder: adenoma	1
B. Mesenchymal neoplasms	
1. Fibrous tissue: fibroma and fibrosarcoma	33
2. Adipose tissue: lipoma	9
3. Myxomatous tissue: myxoma and myxosarcoma	7
4. Cartilage: chondroma	6
5. Bone: osteoma and osteosarcoma	17
6. Smooth muscle: leiomyoma	7
7. Striated muscle: rhabdomyoma and rhabdomyosarcoma	9
8. Blood vessels: hemangioma and hemangioendothelioma	11
9. Lymphatic tissue: lymphosarcoma	16
10. Notochord: chordoma	5
11. Sarcoma: anaplastic, mixed, or unidentified	10
C. Pigment cell neoplasms:	
Melanoma, erythrophoroma, guanophoroma, xanthophoroma	24 + 2 hybrids
D. Neural neoplasms:	
Ganglioneuroma, neuroblastoma, neurilemmoma, neurofibroma, etc	12
E. Teratoma	2

Papillary tumors were also observed on the lips, buccal mucosa, and fins of smelt, *Osmerus eperlanus* (Breslauer, 1916). The fish were collected in brackish water of an inlet of the Baltic Sea. In some instances, there were indications of invasiveness, and cytoplasmic inclusions were sometimes found in the epithelial cells.

Epidermal papillomas have been frequently described in flounders, and several reports indicate a relatively high incidence, often in a particular species and geographic locality. Pacis (1932) and Good (1940) observed many epidermal papillomas of English sole, *Parophrys vetulus*, collected in several parts of Puget Sound, Washington. The fish were generally 1–2 years old. The tumors occurred anywhere on the external surfaces with a preference for the eye side, and some were thought to be invasive. Good (1940) states that 10 of 12 tumors on 11 fish continued to enlarge in the laboratory, and no new tumors appeared in 15 weeks. Attempts to transplant the tumors failed, as did efforts to induce tumors with 3-methylcholanthrene.

TABLE 6.—Osteichthyes: Epithelial tumors of the skin and buccal cavity

Reference	Species	Number of fish	Pathology, comments
McFarland, 1901	*Ictalurus catus* (catfish)	1	Epidermoid carcinoma, lip.
Dauwe and Pennemann, 1904	*Cyprinus carpio* (carp)	2	Epidermoid carcinoma, dorsum of head.
Bashford *et al.*, 1905	*C. carpio*	1	Epidermoid carcinoma.
Murray, 1908	*Spinachia spinachia* (stickleback)	1	Epidermoid carcinoma, tail.
Schroeders, 1908	*Gobio*, several species	Several	Epidermal papillomas, solitary or multiple, of skin.
Keysselitz, 1908	*Barbus fluviatialis*	Many	Epidermal papillomas, lips.
Fiebiger, 1909a	*Anabas scandens* (climbing perch)	Several	Epidermal papillomas, widespread, of skin.
	Tinca tinca (tench)	2	Epidermal papillomas (?) or epidermoid carcinoma (?), lips.
	C. carpio	1	Epidermoid carcinomas, multiple.
Clunet, 1910	*Barbus vulgaris* (barbel)	1	Epidermoid carcinoma, lower lip.
Mazzarelli, 1910	*Chondrostoma soetta* (LeNez)	1	Epidermoid carcinoma (?), buccal cavity.
Johnstone, 1912	*Hippoglossus hippoglossus* (halibut)	1	Epidermal papilloma, snout.
Breslauer, 1916	*Osmerus eperlanus*	37	Epidermal papillomas, lips, oral mucosa, and fins.
Beatti, 1916	*Pogonias chromis* (croaker)	2	Epidermoid carcinoma, lips.
Anitschkov and Pavlovsky, 1923	*Gobio nigronotatus*	2	Epidermal papilloma, skin.
	Gobio blennioides	1	Epidermal papilloma, skin.
Johnstone, 1924	*Merlangus merlangus*	1	Epidermoid carinoma, mandible and buccal cavity.
Johnstone, 1925	*Pleuronectes platessa* (plaice)	1	Epidermal papillomas, skin.
	Limanda limanda (plaice)	3	Epidermal papillomas.
Sagawa, 1925	*Carassius auratus* (goldfish)	2	Epidermal papillomas, skin of pectoral fins. (May represent the normal papillomatous growths which appear on the pectoral fins of male goldfish in the breeding season.)
Takahashi, 1929	*Carassius carassius* (crucian carp)	1	Epidermal papilloma, operculum.
	Theragra chalcogrammus (coal fish)	1	Adenocarcinoma, buccal cavity and operculum.
Williams, 1929	*Pollachius virens*	1	Epidermoid carcinoma (?) or adenoid cystic carcinoma (?).
Puente-Duany, 1930	*Cichlasoma tetracanthus* (biajaca)	1	Epidermoid carcinoma (?), orbit.
Thomas, L., 1930	*Solea solea* (sole)	2	Epidermal papilloma, skin of body.
Thomas, L., and Oxner, 1930	*Anguilla anguilla* (*Anguilla vulgaris*) (European eel)	3	Epidermal papillomas, lips.
Pacis, 1932	*Parophrys vetulus* (English sole)	Many	Epidermal papillomas. (Author thought tumors were adenocarcinomas.)

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Table 6.—Osteichthyes: Epithelial tumors of the skin and buccal cavity—Continued

Reference	Species	Number of fish	Pathology, comments
Thomas, L., 1932e	*Salmo trutta* (brown trout)	1	Epidermal papilloma, dorsum of trunk.
Ladreyt, 1935	*Muraena helena* (moray eel)	1	Adenocarcinoma of mucosal glands, palate.
Smith, G. M., 1935	*Pseudopleuronectes americanus* (flounder)	1	Epidermal papilloma (?), regarded as hyperplastic epidermal disease by the author. Associated with encysted metacercariae of a trematode.
Coates *et al.*, 1938	*Electrophorus electricus* (electric eel)	1	Epidermal papilloma, dorsum of trunk.
Lucké, 1938	*Iridio bivittata* (slippery dick)	30 of 6,000 specimens	Epidermal papillomas, skin.
Good, 1940	*P. vetulus*	61	Epidermal papillomas, skin.
Lucké and Schlumberger, 1941	*Ameiurus nebulosus* (catfish)	166	Epidermal papillomas and epidermoid carcinomas of lips, dental plates, skin.
Nigrelli, 1946	*Macrozoarces americanus*	1	Epidermal papilloma (?), fibroepithelial, of snout.
Christiansen and Jensen, 1947	*A. anguilla*	Epizootic	Epidermal papillomas, usually of head.
Lucké and Schlumberger, 1948	*Catastomus commersonnii* (sucker)	1	Epidermal papillomas (2), caudal peduncle and caudal fin.
Aronowitz *et al.*, 1951	*Xiphophorus variatus*	1	Epidermoid carcinoma, head.
Tavolga, 1951	*Bathygobius soporator* (goby)	4	Epidermoid carcinomas.
Nigrelli, 1952a	*A. scandens*	Several	Epidermal papillomas, skin.
	Colisa lalia (dwarf gourami)	Several	Epidermal papillomas, lip.
Oota, 1959	*Acanthogobius flavimanus* (goby)	Epizootic	Epidermal papillomas.
Sarkar and Dutta-Chaudhuri, 1953	*Anabas testudenius* (Koi-fish)	Several	Epidermal papilloma.
Schaeperclaus, 1953	*A. anguilla*	Many	Epidermal papillomas.
Stolk, 1953c	*Xiphophorus helleri* (swordtail)	?	Epidermoid carcinoma.
Ermin, 1954	Hybrid of *Anatolichthys* × *A. transgrediens*	1	Epidermoid carcinoma, orbit.
Nishikawa, 1954	*A. flavimanus*		Epidermal papilloma.
Stolk, 1956e	*Colis labiosa*	6	Epidermoid carcinoma, skin.
Russell and Kotin, 1957	*Genyonemus lineatus* (white croaker)	10	Epidermal papilloma.
Stolk, 1958c	*Ephippicharax orbicularis*	17	Epidermoid carcinoma.
Imai and Fujiwara, 1959	*A. flavimanus*	1	Epidermal papilloma, skin. Electron microscopy revealed virus-like particles.
Stolk, 1960b	*Etroplus maculatus*	5	Epidermoid carcinoma.

Wellings *et al.*, 1963 (*See also:* Wellings and Chuinard, 1964; Chuinard *et al.*, 1964; Wellings *et al.*, 1964; Wellings, Chuinard, and Bens, 1965; Wellings, Cooper, and Chuinard, 1966; Wellings, Chuinard, and Cooper, 1967; Chuinard, Berkson, and Wellings, 1966.)	*Hippoglossoides elassodon* (flathead sole)	Epizootic, many	*Epidermal papillomas, body, fins, operculum, eyes, buccal cavity.
Young, P. H., 1964	*Microstomus pacificus* (dover sole)	Epizootic	Epidermal papillomas.
Nigrelli *et al.*, 1965	*Psettichthys melanostictus* (sand sole)	Epizootic	Epidermal papillomas.
	Lepidopsetta bilineata (rock sole)	5	Epidermal papillomas.
Wellings *et al.*, 1965	*P. melanostictus*	1	Epidermal papillomas, trunk.
	Glyptocephalus zachirus (rex sole)	3	Epidermal papillomas, trunk and fins.
	P. vetulus	2	Epidermal papillomas, trunk and fins.
Wellings *et al.*, 1966	*Platichthys stellatus* (starry flounder)	Epizootic	Epidermal papillomas, skin, body, fins, eyes, operculum, buccal cavity.
	P. vetulus	Epizootic	Epidermal papillomas, skin, body, fins, eyes, operculum, buccal cavity.
Von Sallmann *et al.*, 1966	*Salmo gairdneri*	87	Cataracts, and possibly epidermoid carcinoma, lens, experimentally produced by thioacetamide added to the diet.
Kimura, Sugiyama, and Ito, 1967	*Limanda herzensteini* (flounder)	1	Epidermal papillomas.
	Hippoglossoides dubius (flounder)	4	Epidermal papillomas.
Kimura, Miyake, and Ito, 1967	*A. flavimanus*	?	Epidermal papillomas.
McArn *et al.*, 1968	*P. vetulus*	Epizootic	Epidermal papillomas, 1–104 tumors per fish.
	P. stellatus	Epizootic	Epidermal papillomas, 1 to several per fish.
Mawdesley-Thomas and Bucke, 1967	*Gobio gobio*	1	Epidermoid carcinoma.
Wellings, S. R., unpublished	*M. pacificus*	2	Epidermal papillomas, 1 tumor per fish.
	L. bilineata	17	Epidermal papillomas, 1–8 tumors per fish.
Honma and Kon, 1968	*Glyptocephalus stelleri*	1	Epidermal papilloma.

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*There were three morphological forms of these tumors, apparently histogenetically related. All are recorded as epidermal papillomas for the present purpose.

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Tumors in flounders have also been described in epizootic proportions in the flathead sole *Hippoglossoides elassodon* (Wellings *et al.*, 1963, 1964). From July 1963 to July 1964, 281 tumor-bearing fish were observed in a total of 4,364 fish (6.4% tumor incidence). The evidence indicated that the tumors originated in the first year of life as small, 1–2 mm fibrovascular proliferations in the dermis, capped by a layer of hyperplastic epidermis. These lesions evolved into typical epidermal papillomas near the end of the first year. It was also observed that the number of tumors per fish was greatest in the first year of life, and decreased thereafter. Although tumors occurred anywhere on the outer surfaces of the fish, there was a significantly greater incidence on the eye side.

Regular collections of flathead sole have been made in East Sound of Orcas Island, Washington, from 1963 to the present, and the tumor incidence remains between about 4 and 6% of the total collection, now numbering many thousands. Several attempts to transplant the tumors, both autologously and homologously, have failed, as have efforts to transmit the disease to fish older than 1 year by means of cell-free homogenates and filtrates. Virus-like particles are often observed in electron micrographs of the tumor cells. Similar tumors have now been observed in several other species of flounders by the same authors (fig. 1) (*see* reports listed in table 6) and by P. H. Young (1964), Nigrelli, Ketchen, and Ruggieri (1965), and Kimura, Sugiyama, and Ito (1967).

Recently McArn *et al.* (1968) described a similar phenomenon among starry flounders (*Platichthys stellatus*) and English sole (*Parophrys vetulus*) collected in Bellingham Bay, Port Susan, and Port Gardner of Puget Sound, Washington. Again the incidence is greatest in the young fish of the year. As many as 50% of starry flounders (age group 0) in some collections from the estuary of the Nooksack River are affected.

Starry flounders collected at several other sites (Wellings and McArn, unpublished) showed no tumors, suggesting that undefined environmental factors are of primary importance in tumorigenesis. Young fish, both tumor-bearing and non-tumor-bearing, at all collecting sites are usually infested by trematodes of the genus *Gyrodactylus* (fig. 2). There is as yet no evidence that these or any of several other incident ectoparasites function as vectors. Elucidation of the cause and natural history of epidermal papillomas in flounders will probably require studies of the total biology of the species affected.

There is some doubt as to whether the lesions of the flounder papillomas are truly neoplastic. Nigrelli, Ketchen, and Ruggieri (1965) tend to regard the lesions as a hyperplastic epidermal disease and indicate morphological evidence that the tumors are regressive processes rather than progressive neoplasia. Nigrelli has noted on a number of occasions that epidermal hyperplasia in several species of fishes can be associated with various parasitic infestations (1948a, 1948b, 1954, 1959). Among the parasites implicated are myxosporidians, microsporidians, ciliates, nematodes, and trematodes. In the literature it also seems apparent that the term "fish pox" has been used interchangeably on occasion with both "hyperplastic epidermal disease" and "epidermal papilloma." Fish pox, of unknown etiology, may be caused by multiple etiologic agents, including viruses. It seems impossible at present to decide whether the common type of proliferative lesion of flounder epidermis is hyperplastic or neoplastic. In any case, the lesion sufficiently resembles neoplasia to be of considerable interest to the cancer biologist.

A helpful statement by Schlumberger and Lucké (1948) is: "On histological grounds no sharp distinction can be drawn between these lesions [pox and hyperplastic epidermal disease] and true neoplasms, but clinically the lesions of pox, unlike true tumors, usually regress and disappear entirely." So far, the epidermal tumors of flathead sole and starry flounders have not regressed in the laboratory; on the other hand, we have not been able to transplant them successfully.

Lucké (1938) observed 30 fish with epidermal papillomas in a collection of approximately 6,000 slippery dick, *Iridio bivittata*, examined at the Dry Tortugas, Florida.

In 1941, Lucké and Schlumberger described a transplantable epithelioma, sometimes invasive, on the lips and in the buccal cavity of about 200 catfish, *Ameiurus nebulosus*, collected in heavily polluted water near Philadelphia. The early lesions consisted of a focal zone of intense hyperemia of the skin. This was followed by epidermal thickening and finally by the development of epidermoid carcinoma. Some were deeply invasive and emboli of tumor cells were found in vessels of the larger tumors. Autotransplants and homotransplants to the anterior chamber of the eye, and autotransplants to the cornea, were successful.

Epidermal papillomas were observed on eels, *Anguilla anguilla*, captured in the waters between Sweden and Denmark and in the Baltic Sea (Christiansen and Jensen, 1947; Schaeperclaus, 1953). Most of the papillomas involved the head, but sometimes occurred elsewhere, and many were bulky. The high incidence of the disease suggests an infectious process.

Aronowitz, Nigrelli, and Gordon (1951) described a spontaneous epidermoid carcinoma on the dorsolateral surface of the head of a platyfish, *Xiphophorus variatus*. Microscopically, there were epithelial pearls and invasion of adjacent structures.

It has been common knowledge for some years that formations indistinguishable from neoplastic papillomas normally appear on pectoral fins and opercula of male goldfish, *Carassius auratus*, during the breeding season. An experimental analysis of this phenomenon reveals it to be androgen-dependent (Ghadially and Whiteley, 1952). It seems possible that the report of Sagawa (1925) represents the same normal physiological phenomenon, rather than neoplasia.

Nigrelli (1952a) reported epidermal papillomas on climbing perch, *Anabas scandens*, and on the lips of 0.5–1% of dwarf gourami, *Colisa lalia*. In the last species, the disease occurred when the fish were raised in outdoor pools, and there was evidence of inclusion bodies in the affected epithelial cells. Although attempts to transmit the disease in the laboratory failed, under natural conditions the epidemiological evidence suggested an infectious process.

On numerous occasions, epidermal papillomas have been described in the Japanese goby *Acanthogobius flavimanus* (Oota, 1952; Nishikawa, 1954; Iwashita, 1955; Kimura, Miyake, and Ito, 1967). Apparently the disease is epizootic. Virus-like particles, observed by electron microscopy in the affected epithelial cells, bear partial resemblance to the virus of molluscum contagiosum (Imai and Fujiwara, 1959).

Stolk (1953c) observed epidermoid carcinomas in 11 specimens of the black variety of *Xiphophorus helleri.* The tumors originated near the base of the tail and infiltrated the corium, subcutaneous tissue, and muscle. The neoplastic epidermal cells were pleomorphic and there was an accompanying overgrowth of melanophores.

Stolk (1956e) also observed skin carcinomas in six siblings of the anabantid *Colis labiosa.* The tumors were locally invasive, lobulated, sessile, nodular masses.

Squamous (epidermal) papillomas were observed on 10 white croakers, *Genyonemus lineatus*, from a collection of 353 fish of the same species (Russell and Kotin, 1957). The tumors were multiple, small, firm, white lesions, located about the mouth. Microscopically, there were thickened epithelium, intact basement membranes, and occasional epithelial pearls of nonkeratinized epithelial cells. The fish were collected in marine waters 2 miles from a sewage outfall in Los Angeles harbor. A total of 1,116 white croakers were collected from an unpolluted area 50 miles away, but no tumors were found. The authors suggested a possible role of pollution.

An epithelial lesion was observed on the upper lip in 17 (14 males, 3 females) *Ephippicharax orbicularis* (Stolk, 1958c). The tumors were partially papillary, and the stroma was composed of vascular connective tissue. There "appeared to be little invasion" and basement membranes were intact. The author diagnosed the tumor as epidermoid carcinoma.

Epidermoid carcinomas were noted in 5 male *Etroplus maculatus* (Stolk, 1960b). The tumors were large and crater-shaped. The neoplastic epithelial cells were closely packed and supported by a delicate, richly vascular connective tissue stroma. Nuclei were pleomorphic with coarse chromatin patterns, and the tumors had invaded the corium and muscle locally. A sex-linked genetic basis was proposed by the author.

A-2. Dental Tumors

Dental tumors have been observed in five species of bony fish (table 7).

Among the more recent reports, Schlumberger and Katz (1956) described multiple odontogenic tumors (fibroameloblastomas) on the jaws of two chinook salmon, *Oncorhynchus tshawytscha*, collected in the Columbia River in May 1955. The tumors were located on the dental surfaces of the jaws and on accessory dental surfaces (vomer, glossohyal, basibranchials, and branchial arches). The masses were bulky and prevented complete closure of the mouth. Microscopically, there were irregular

TABLE 7.—Osteichthyes: Dental tumors

Reference	Species	Number of fish	Pathology, comments
Plehn, 1915	*Salmo fario* (European brook trout)	2	Multiple odontomas.
Roffo, 1925	*Micropogon opercularis* (croaker)	1	Odontoma.
Thomas, L., 1926	*Melanogrammus aeglifinus* (haddock)	1	Ameloblastoma.
Schlumberger and Katz, 1956	*Oncorhynchus tshawytscha* (chinook salmon)	2	Fibroameloblastomas.
Stolk, 1957i	*Cyprinodon variegatus variegatus*	1	Ameloblastomas, multiple, of jaws.

proliferations of epithelial cells supported by abundant connective tissue stroma. In tumors of the tooth-bearing regions, the epithelial component formed islands of stellate reticulum surrounded by palisaded columnar cells, thus resembling the typical ameloblastomas of mammals.

Ameloblastomas were described on the jaws of a *Cyprinodon variegatus variegatus* (Stolk, 1957i). There were multiple nodules on both jaws composed of nests of epithelial cells in a connective tissue stroma. The histology was typical of that of ameloblastomas in mammals.

A–3. Pharynx, Parabranchial Bodies, and Gills

Epithelial tumors of the pharynx, pharyngeal glands, parabranchial bodies,[2] and gills are described in eight species, as indicated in table 8.

TABLE 8.—Osteichthyes: Epithelial tumors of pharynx, parabranchial body, and pharyngeal gland

Reference	Species	Number of fish	Pathology, comments
Peyron and Thomas, 1929	*Gadus morhua* (codfish)	2	Adenomas, parabranchial body; one case unilateral, other bilateral.
Takahashi, 1929	*Pollachius brandti* (codfish)	2	Adenocarcinoma, parabranchial body; one case unilateral, other bilateral.
	Theragra chalcogrammus (coal fish)	1	Adenocarcinoma, parabranchial body.
Stolk, 1953d	*Lebistes reticulatus* (guppy)	1	Carcinoma, pharynx.
Stolk, 1957g	*Haplochromis multicolor*	1	Adenoma, pharyngeal gland.
Stolk, 1962	*Betta anabatoides*	2	Adenocarcinoma and adenoma, pharyngeal gland.
Sarkar and Dutta-Chaudhuri, 1964	*Salmo fario* (trout)	1	Adenoma, gill.
Wellings, S. R., unpublished	*Gadus macrocephalus* (codfish)	28	Adenomas (?), parabranchial body, bilateral.

Adenomas (or adenocarcinomas?) of the parabranchial bodies (pseudobranch) appear to be characteristic of cod and related fishes, and have been observed in *Gadus morhua* (Peyron and Thomas, 1929), *Pollachius brandti*, *Theragra chalcogramma* (Takahashi, 1929), and apparently in *Gadus macrocephalus* (Wellings, S. R., unpublished). In the last species, the tumors were observed in 28 codfish, were always bilateral, and were located in the superiolateral pharynx, anterior to the true gills, and hidden by the opercula. The tumors varied from 2 × 2 × 1 cm to 6 × 4 × 3 cm, were lobulated, and protruded into the pharyngeal cavity (fig. 3). Microscopically, the neoplastic cells formed sheets of loosely aggregated cells with increased nuclear-cytoplasmic ratio, and with prominent nucleoli (fig. 4). Tissue interpreted as possible normal elements of a preexisting glandular structure was observed in the center of some tumors (fig. 5).[2]

A carcinoma of the pharynx in a female guppy, *Lebistes reticulatus*, was observed by Stolk (1953d). The neoplasm originated from the ventral wall of the pharynx and infiltrated the gills, ventral musculature of the body wall, and the connective tissue found caudal to the gill regions. Microscopically, the tumor cells resembled pharyngeal epithelial cells, from which they apparently arose.

An adenocarcinoma of the pharyngeal gland in an adult female *Haplochromis multicolor* was also observed by Stolk (1957g). The tumor, located on the dorsal surface of the palate, consisted of an irregular, nodular mass. Microscopically, a mixture of alveolar and nonalveolar epithelial formations was supported in a connective tissue stroma. Tumor cells infiltrated the local connective tissue and muscle. Stolk (1957f) described the normal pharyngeal glands.

Neoplasms of the pharyngeal glands were described in two specimens of *Betta anabatoides* (Stolk, 1962). An adenocarcinoma occurring on the dorsal surface of the palate of an adult male infiltrated adjacent fibromuscular tissues. An adenoma was also observed in a young male; in this instance there was no infiltration.

A-4. Stomach and Intestine

The three instances of epithelial neoplasia in the stomach and intestine of bony fishes found in the literature (table 9) were reviewed by Schlumberger and Lucké (1948). Nigrelli (1947a) reported a "fibro-carcinoma-like growth" in the stomach of a specimen of *Acentrophryne* (?) *longidens*. Microscopically, there was massive overgrowth of connective tissue elements and cells of the mucosa with stenosis, especially in the pyloric region. Epithelial-like cells diffusely infiltrated portions of the gastric wall. The description suggests that the tumor was essentially identical to scirrhous carcinoma of the human stomach.

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[2] I have been unable to locate detailed descriptions of "parabranchial bodies" in the literature. However, a very brief description may be found in Schlumberger and Lucké (1948). The tumors of *Gadus macrocephalus* described in the text correspond closely to the description of tumors of the parabranchial bodies in the manuscripts of Peyron and Thomas (1929) and Takahashi (1929) and are believed to be identical.

TABLE 9.—Osteichthyes: Epithelial tumors of the stomach and intestine

Reference	Species	Pathology, comments
Takahashi, 1929	*Theragra chalcogramma*	Adenocarcinoma, digitiform (rectal) gland.
Thomas, L., 1931a	*Salmo mykiss*	Cystic adenoma, intestine.
Nigrelli, 1947a	*Acentrophryne* (?) *longidens*	"Fibrocarcinoma," possibly scirrhous adenocarcinoma, stomach.

A-5. Liver Tumors

Primary hepatic neoplasms generally arise from either the epithelium of the bile ducts (cholangioma and cholangiocarcinoma) or from the hepatic parenchymal cells (hepatocellular adenoma and hepatocellular carcinoma).

Since hepatic neoplasms of salmonids constitute the vast majority of reports of liver tumors in the bony fishes, the following survey of the literature will be conducted under two headings: *a*) and *b*) hepatic neoplasia in salmonids (tables 10A and 10B), and *c*) hepatic neoplasia in bony fishes other than salmonids (table 11), of which there are two instances in the literature.

a) *Hepatic neoplasia in salmonids: Reports from 1909–60* (*table 10A*)

Plehn (1909) first reported a liver tumor in a salmonid fish. He described a multilocular cystadenoma, apparently of cholangiolar origin, in the liver of a brown trout, *Salmo trutta.* In 1933, Haddow and Blake observed a hepatocellular adenoma in a rainbow trout *Salmo gairdneri.* Since 1953, there have been scattered reports of hepatocellular adenomas and carcinomas in rainbow trout (Scolari, 1953; Nigrelli, 1954; Nigrelli and Jakowska, 1955; Cudkowicz and Scolari, 1955; Levaditi *et al.*, 1960) from the United States, Africa, and Europe. Honma and Shirai (1959) reported a monolocular cystadenoma of the liver of a rainbow trout.

TABLE 10A.—Osteichthyes: Hepatic neoplasia in salmonids (reports from 1909–60)

Reference	Species	Number of fish	Pathology, comments
Plehn, 1909 and 1924	*Salmo trutta* (brown trout)	1	Cystadenoma, liver.
Haddow and Blake, 1933	*Salmo gairdneri* (rainbow trout)	1	Hepatocellular adenoma.
Scolari, 1953	*S. gairdneri*		Hepatocellular carcinoma.
Nigrelli, 1954	*S. gairdneri*	1	Hepatocellular carcinoma.
Nigrelli and Jakowska, 1955	*S. gairdneri*	4	Hepatocellular carcinoma, 4 cases in 600 3-year-old rainbow trout from a Pennsylvania hatchery.
Cudkowicz and Scolari, 1955	*S. gairdneri*	Many	Hepatocellular carcinoma, many cases from fish hatcheries in northern Italy.
Honma and Shirai, 1959	*S. gairdneri*	1	Cholangiolar cystadenoma.
Levaditi *et al.*, 1960	*S. gairdneri*		Hepatocellular carcinoma.

Table 10B.—Hepatic neoplasms in salmonids: Reports dealing with the high prevalence, geography, biology, and etiology of the disease, 1960–67; rainbow trout, *Salmo gairdneri;* cutthroat trout, *Salmo clarki;* brook trout, *Salvelinus fontinalis;* and brown trout, *Salmo trutta*

Reference	Abstract
Ashley and Halver, 1961	Evidence that certain dry, pelleted feeds, when fed to rainbow trout for 9–15 months, produced a 50% incidence of primary hepatomas.
Ghittino, 1961	Reports of hepatoma in rainbow trout in Europe.
Hueper and Payne, 1961	Observed many hepatocellular carcinomas and one mixed hepatocellular and cholangiocarcinoma among 143 hatchery-reared rainbow trout. No similar pathology was found in a 1- to 2-year-old brown trout or in 73 brook trout. The brown trout had a 1 cm cavernous hemangioma in the liver.
Nigrelli and Jakowska, 1961	Examined 333 trout livers from hatcheries located mainly in Idaho, Montana, and Wyoming. Most specimens were from rainbow trout, but a few were from brown trout. Fifty cases of nodular hyperplasia were found, and 19 livers were diagnosed as neoplastic, with the alterations ranging from adenoma to hepatocellular carcinoma.
Rucker *et al.*, 1961	Discusses trout hepatoma in relation to possible environmental and nutritional variables.
Snieszko, 1961	Hepatoma in trout.
Wood and Larsen, 1961	Found an incidence of nodular neoplastic livers ranging from 10–75% of fish in several fish hatcheries in Idaho, Montana, Utah, and Washington. The morphology ranged from hepatocellular adenoma to hepatocellular carcinoma.
Ashley *et al.*, 1962	Demonstrated that a potent hepatomagenic agent was present in the lipid fraction extracted from a dry, pelleted, commercial trout feed suspected of being hepatomagenic. Aminoazotoluene induces cholangiolar carcinoma.
Halver *et al.*, 1962	Diets, with added aminoazotoluene, DDT, *p*-dimethylaminoazobenzene, diethylstilbestrol, and dimethylnitrosamine, are hepatomagenic.
LaRoche *et al.*, 1962	Changes suggestive of early hepatomagenesis can be produced by the lipid fraction extracted from a known hepatomagenic, dry, pelleted, commercial trout feed.
Ashley and Halver, 1963	Demonstration of multiple metastases of rainbow trout hepatocellular carcinomas.
Levaditi *et al.*, 1963	Discussed the appearance of hepatic carcinomas in rainbow trout in France, Italy, and the United States since 1953. Proposed a genetic and nutritional basis for the disease.
Scarpelli *et al.*, 1963	Described 11 fish with hepatic adenomas, and 28 instances of hepatoma (hepatic carcinoma). The majority were hepatocellular carcinomas; others were of a spindle cell type. A cholangiohepatoma consisted of neoplastic cells of both cholangiolar and hepatocellular types. Most of the tumors were well differentiated, and only 4 of the 28 tumors had metastasized. On the basis of morphologic and enzyme patterns, may be classified as minimal deviation hepatomas.

TABLE 10B.—Hepatic neoplasms in salmonids: Reports dealing with the high prevalence, geography, biology, and etiology of the disease, 1960–67; rainbow trout, *Salmo gairdneri*; cutthroat trout, *Salmo clarki*; brook trout, *Salvelinus fontinalis;* and brown trout, *Samlo trutta*—Continued

Reference	Abstract
Wolf and Jackson, 1963	Demonstrated that the omission of cottonseed meal from a known hepatomagenic trout diet results in reduction of tumor incidence from 48 to 0%. Thus, a potent hepatomagenic carcinogen was present in some samples of cottonseed meal.
Ashley *et al.*, 1964	Rainbow trout fed crude aflatoxins at 0.8 ppm of diet developed classical multinodular hepatoma in almost 60% of livers. Fish force-fed high levels (0.03–0.3 mg/kg/day) of purified aflatoxin B_1 and G_1 for 5 days died with acute aflatoxicosis.
Ashley *et al.*, 1965	Classical trabecular hepatomas developed in trout fed purified crystalline aflatoxins B_1 and G_1. High loads of aflatoxin B_1 produced acute aflatoxicosis, similar to that produced by the crude aflatoxin.
Halver, 1965	Relationships between aflatoxicosis and rainbow trout hepatoma.
Sinnhuber *et al.*, 1965	Aflatoxin-containing commercial trout rations, cottonseed meal, solvent extracts of cottonseed meal, and aflatoxin, when added to a purified test diet, resulted in hepatocarcinoma in 9 months.
Halver and Mitchell, eds., Bureau of Sport Fisheries, Research Report #70, 1967, 199 pp.	Progress reports, historical perspective, biology, experimental analysis, and pathology of trout hepatomas.

b) Hepatic neoplasia in salmonids: Reports from 1960–67

In 1960 it became apparent that hepatocellular carcinoma had reached epidemic proportions (up to almost 100% of the fish affected) in Federal, State, and private fish hatcheries at widely scattered localities throughout the United States, and that the cutthroat trout, *Salmo clarki*, and brown trout, *Salmo trutta*, were also affected. The basic references, which establish the pathology, prevalence, and geography of the disease, are listed in table 10B (Ashley and Halver, 1961; Ghittino, 1961; Hueper and Payne, 1961; Nigrelli and Jakowska, 1961; Rucker, Yasutake, and Wolf, 1961; Snieszko, 1961; Wood and Larsen, 1961).

The pathology and biology of the disease are described in a number of further reports (Ashley, Halver, and Johnson, 1962; Halver, Johnson, and Ashley, 1962; LaRoche, Halver, Johnson, and Ashley, 1962; Ashley and Halver, 1963; Levaditi *et al.*, 1963; Scarpelli, Greider, and Frajola, 1963; Wolf and Jackson, 1963; Ashley, Halver, and Wogan, 1964; Ashley *et al.*, 1965; Halver, 1965; Sinnhuber *et al.*, 1965) and in a recent summary published by the Bureau of Sport Fisheries (Trout Hepatoma Research Conference Papers, Research Report #70, Halver and Mitchell, eds., 1967).

Table 11.—Osteichthyes: Liver tumors in bony fishes other than salmonids

Reference	Species	Number of fish	Pathology, comments
Dawe *et al.*, 1964	*Catastomus commersoni* (white sucker)	3	2 cholangiomas, 1 cholangiocarcinoma.
	Ictalurus nebulosus (brown bullhead)	1	Hepatocellular carcinoma, well differentiated (*i.e.*, minimal deviation hepatoma).
Stanton, 1965	*Brachydanio rerio*	17	Hepatocellular adenoma, hepatocellular adenocarcinoma. Cholangioma, cholangiocarcinoma, all experimentally produced by the addition of diethylnitrosamine to the water.

Since the present paper does not extensively review particular instances of neoplasia, suffice it to say that the hepatocellular carcinomas occurring in hatchery-reared trout in the past decade are probably related to liver damage produced by the ingestion of aflatoxins, formerly in some dry, pelleted commercial feeds, particularly those containing a high percentage of cottonseed mash. The responsible aflatoxins, of several molecular species, are synthesized by certain mutants of *Aspergillus flavus*, capable of growth in high lipid, vegetable, dietary constituents (such as cottonseed and peanuts) when these are stored under damp conditions in the field or in warehouses. The story of the solution of the trout hepatoma problem is a dramatic one and a tribute to the cooperative efforts of many talented persons.

c) *Hepatic neoplasia in bony fishes other than salmonids*

The report of Dawe, Stanton, and Schwartz (1964) described hepatic neoplasms occurring in two species of bottom-feeding fish collected from Deep Creek Lake, Maryland. Three of a sample of 12 white suckers, *Catostomus commersoni*, had hepatic neoplasms, 2 of which appeared to be cholangiomas and 1 an invasive cholangiocarcinoma. One of a sample of 100 brown bullheads, *Ictalurus nebulosus*, displayed multiple liver nodules composed of proliferations of hepatic parenchymal cells. These tumors were interpreted as probable minimal deviation hepatomas. Four other species of fish were without neoplasms.

Stanton (1965) induced liver tumors in *Brachydanio rerio* by adding diethylnitrosamine to the aquarium water. Initially, necrosis of liver cells occurred, followed by the appearance of multiple foci of regenerating hepatic and biliary cells. In 17 of 63 fish, macroscopic hepatomas and cholangiomas, sometimes invasive, were observed between the 10th and 30th weeks of the experiment. This work is important because hepatomagenesis in teleosts can now be conveniently analyzed under laboratory conditions without elaborate facilities for maintenance of the fish.

A-6. Pancreas

There are only two reports of epithelial neoplasms arising in the pancreas of bony fishes. Nigrelli and Gordon (1952) observed an acinar adenocarcinoma in a 16-month-old male hybrid platyfish, *Xiphophorus variatus* × *Xiphophorus xiphidum*. The larger part of the neoplasm formed a mass between the folds of the intestine. In some portions of the tumor the epithelial cells formed acini, and the cytoplasm of the neoplastic cells contained zymogen granules which were sometimes also observed in the tissue spaces. Varying degrees of nuclear anaplasia were noted and necrosis and hemorrhage were extensive. Although the neoplastic tissue had invaded surrounding structures and had apparently completely destroyed the endocrine pancreas and the testis, there were no metastases.

Otte (1964) described an adenocarcinoma of the pancreas, about the size of an egg, in a goldfish, *Carassius auratus*. The neoplasm was attached to the intestine, had metastasized to the mesentery, and there were central necrosis and hemorrhage. Microscopically, there were cords and nests of neoplastic epithelial cells with increased nuclear-cytoplasmic ratio, nuclear hyperchromatism, nuclear pleomorphism, increased mitoses, and atypical mitoses. Zymogen granules were observed in the cytoplasm of some of the cells.

A-7. Kidney

There are at least four reports of neoplasia of the kidneys (table 12). Several other references were omitted because of incompleteness.

Schlumberger and Lucké (1948) observed an adenoma in the right kidney of a female catfish, *Ameiurus nebulosus*, of 35 cm total length. The neoplasm was 8 × 6 × 8 mm, pale gray, and well circumscribed. Microscopically, the mass contained acinar spaces, sometimes dilated or with papillary internal projections. The neoplastic epithelial cells were low cuboidal to tall columnar with large vesicular nuclei, prominent nucleoli, and infrequent mitoses. It was of interest that the fish also had massive epitheliomas of the lip and mouth.

TABLE 12.—Osteichthyes: Kidney tumors

Reference	Species	Number of fish	Pathology, comments
Schmey, 1911	*Anguilla anguilla* (eel)	1	Adenocarcinoma, left kidney.
Schlumberger and Lucké, 1948	*Ameiurus nebulosus* (catfish)	1	Adenoma, right kidney (this specimen also had massive epithelial neoplasms of the lip and mouth).
Stolk, 1957a	*Thayeria obliqua*	1	Adenocarcinoma.
Stolk, 1957d	*Barbus tetrazona*	1	Adenocarcinoma.
Sathyanesan, 1966	*Mystus vittatus* (catfish)	1	"Glomerular cystic tumor" [developmental defect (?)].
Ashley, 1967	*Salmo gairdneri* (rainbow trout)	Many	Nephroblastoma, experimentally produced by dimethylnitrosamine added to the diet.

An adenocarcinoma of the kidney was observed in an adult female *Thayeria obliqua* by Stolk (1957a). The tumor was large and filled most of the coelomic cavity, displacing the intestine, liver, and ovaries. Microscopically, there were strands and masses of tumor cells with pleomorphic nuclei. Most of the kidney was replaced by tumor, and there was invasion of muscle. The author felt there was some resemblance to human hypernephroma (adenocarcinoma of the kidney).

Another case of adenocarcinoma, also described by Stolk (1957d), arose in the kidneys of a cyprinid, *Barbus tetrazona*. The tumor filled most of the coelom and compressed or surrounded adjacent organs. Histopathologically, there were tubular and acinar formations and solid strands of cells. Mitoses were frequent. The neoplastic cells had invaded the lateral abdominal musculature.

Sathyanesan (1966) described a distinctive renal enlargement in an adult female catfish *Mystus vittatus*. Multiple cystic spaces developed *inside* the renal glomeruli, affecting approximately 75% of the glomerular population. The cysts were sometimes quite large, or exhibited hyperplasia, sometimes of papillary type, and there was lymphocytic infiltration of the stroma. It seems unlikely that this lesion is a neoplasm; the description suggests a developmental defect.

A-8. Urinary Bladder

There is one report of a carcinoma involving the urinary bladder of a goldfish *Carassius auratus* (Plehn, 1909). The bladder neck was obstructed by the growth, and consequently the bladder itself was greatly distended with fluid. The author observed invasive neoplastic cells which were arranged in alveolar formations. The description suggests that the tumor was an adenocarcinoma.

A-9. Ovary

There are four reports of neoplasia arising in ovaries of bony fishes. A fibroma of the ovary was described in *Carassius vulgaris* by Freudenthal (1928). An adenocarcinoma of the ovary in a pike, *Esox lucius*, was reported by Haddow and Blake (1933) who suggested that the tumor arose from germinal epithelium. The tumor was poorly preserved, measured 3 × 7 cm, and occupied part of the left ovary. No illustrations accompanied the report.

Ovarian teratomas were observed by Stolk (1953a, 1959a) in the guppy *Lebistes reticulatus* and the platyfish *Xiphophorus helleri*. These are described in the section on teratomas.

A-10. Testis

Li and Baldwin (1944) noted interstitial cell tumors in the testis of 3 of a group of 40 adult male swordtails, *Xiphophorus helleri*, that had received intraperitoneal injections of sesame oil. In 2 of the fish the tumors were invasive and thought to be interstitial cell carcinomas, and 1 of the 2 had metastasized to the mesorchium. The individual cells were oval or polygonal with vesicular nuclei; mitoses were rarely observed. In the third fish, the lesion of the testis was diagnosed as local nodular hyperplasia of the interstitial cells.

Nigrelli and Jakowska (1953) observed a large tumor, judged to be a spermatocytoma (seminoma), in the testis in an African lungfish *Protopterus annectens*, which had been in captivity for about 5 years. The tumor measured 50 × 40 × 20 mm and had invaded adjacent viscera, including the cloaca, large intestine, and rogenital system. The tumor cells were polygonal with prominent nucleoli, thus resembling spermatogonia or primary spermatocytes.

A-11. Thyroid

The pathobiology of thyroid enlargements in man and animals is a difficult and confusing subject. Most solitary tumors are either adenomas or adenocarcinomas. Some of the follicular adenocarcinomas are so well differentiated as to be indistinguishable from normal or hyperplastic gland. This fact accounts for such anomalous terminology as "benign metastasizing adenoma." Generally speaking, diffuse enlargement of the thyroid represents non-neoplastic change, most often either hyperplasia or thyroiditis. Multinodular follicular formations in the thyroid are also generally non-neoplastic ("involutionary nodular goiter") and may be the result of an unexplained, uneven response of the glandular parenchyma to stimulation by thyrotropic hormone. Such multinodular hyperplasia is characteristic of several endocrine target organs in mammals, *e.g.*, adrenal, prostate, and mammary glands.

Unfortunately, the well-known interpretive difficulties that pertain to thyroid enlargements in mammals also apply to the numerous reports in fishes. The diffuse distribution of thyroid tissue in fishes adds to the difficulty of establishing whether proliferating and enlarging thyroid tissue is truly invasive and metastasizing, and therefore neoplastic. Furthermore, an unusual feature of thyroid hyperplasia in teleosts is the marked tendency for the epithelial cells to invade and infiltrate surrounding structures, including pharyngeal muscle, bone, gill filaments, and tongue (Gorbman and Gordon, 1951). It seems reasonably certain that a great many of the prior reports of adenoma and adenocarcinoma in the thyroid glands of fishes were actually examples of diffuse hyperplasia or endemic (colloid) goiter. In some, but not all, instances of thyroid enlargement in artificially raised fishes, the hyperplasia subsides following addition of iodine to the diet or water, or following an increase in the supply of fresh, well-oxygenated water (Marine and Lenhart, 1910a, 1910b, 1911; Marine, 1914).

In table 13, the available literature is reviewed, and an attempt is made to make a reasonable judgment as to the nature of the disease described. The interpretation given is not always that of the author, nor are most of the interpretations offered with a conviction of certainty. A number of

TABLE 13.—Osteichthyes: Epithelial tumors of the thyroid gland

Reference	Species	Number of fish	Probable nature of pathology, comments
Bonnet, 1883	*Salmo lacustris* (lake trout)	Over 3,000	Hyperplasia.
Gilruth, 1902	*Salmo gairdneri* (rainbow trout)	3	Hyperplasia.
	Salmo salar	1	Hyperplasia.
Plehn, 1902	Trout, several species	Many	Hyperplasia.
Jaboulay, 1908	Trout, several species	6	Hyperplasia.
Smith, H. M., 1909	*S. lacustris*	1	Possibly adenocarcinoma.
Marine and Lenhart, 1910a, 1910b, 1911, and Marine, 1914	*Salvelinus fontinalis* (brook trout)	Many	Hyperplasia.
Gaylord and Marsh, 1914	Trout, several species	Thousands	Hyperplasia. Occurred in U.S. Government hatcheries. Two instances, possibly adenocarcinoma with metastases.
Marsh and Vonwiller, 1916	*Serranus scriba* and *S. cabrilla* (sea bass)	5	Hyperplasia.
Johnstone, 1924	*Box vulgaris* (porgy)	1	Hyperplasia.
Schreitmüller, 1924	*Barbus fluviatialis* (barbel) *Jordanella floridae* *Clarias dumerlii*		Adenoma or adenocarcinoma vs. hyperplasia.
Leger, 1925	*S. gairdneri*		Possible adenomas and adenocarcinomas, one with metastasis to the heart.
Hamre and Nichols, 1926	Trout fry, species not given		Hyperplasia with exophthalmos. Responded to iodine.
Klemm, 1927	*Danio albolineatus* *Haplochilus chaperi*		Hyperplasia.
Smith *et al.*, 1936	*Rasbora lateristriata* *Heterandria formosa*		Hyperplasia.

Smith, G. M., and Coates, 1937	*R. lateristriata*		Hyperplasia.
Schlumberger and Lucké, 1948	*Echeneis naucrates* (pilot fish)	15	Hyperplasia.
Aronowitz *et al.*, 1951	*Xiphophorus variatus* (platyfish)	1	Hyperplasia.
Gorbman and Gordon, 1951	*Xiphophorus montezumae* (swordtail)	Many	Hyperplasia. Inbred strain.
Nigrelli, 1952	*Fundulus heteroclitus* (killifish)	7	"Interacinous adenoma."
	Cyprinodon variegatus (sheepshead minnow)	10	"Interacinous adenoma."
	Angelichthys isabelita (blue angelfish)	1	Adenocarcinoma.
			All of the above tumors were considered malignant.
Berg *et al.*, 1953	*X. montezumae*	Many	Hyperplasia. Inbred strains.
Robertson and Chaney, 1953	*S. gairdneri*	Many	Hyperplasia in natural population in Great Lakes area.
Baker *et al.*, 1955	*Xiphophorus maculatus*, inbred strains	Many	Possibly adenocarcinoma.
Schlumberger, 1955	*Leponis megalotis megalotis* (large-eared sunfish)	Several	Hyperplasia.
Stolk, 1955b	*Xiphophorus helleri*	Many	Hyperplasia following thiouracil treatment.
	Lebistes reticulatus (guppy)	Many	Hyperplasia following thiouracil treatment.
	X. helleri	6	Hyperplastic adenoma.
	L. reticulatus	9	Hyperplastic adenoma.
Stolk, 1956b	*Tanichthys albonubes*	1	Adenocarcinoma (?).
Stolk, 1956d	*Chiclasoma biocellatum*	1	Adenocarcinoma (?).
MacIntyre, 1960	*Girardinus falcutus*	1	Hyperplasia. Relieved by iodine.
	Xiphophorus variatus variatus	1	Hyperplasia vs. adenocarcinoma. This tumor showed extensive growth in the pharyngeal area, spleen, and body cavity.
MacIntyre and Baker-Cohen, 1961	*Xiphophorus variatus xiphidium* (spike-tailed platyfish)	1	Hyperplasia.
Woodhead and Ellett, 1967	*L. reticulatus*	Several	Hyperplasia vs. adenoma.

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reports have been omitted, usually because of incompleteness, lack of illustrations, failure to identify the species involved, or unavailability of the manuscript. Persons desiring to make judgments in this area of fish pathology should study the cited literature with care.

The interesting observations of Hamre and Nichols (1926) are noteworthy. These observers described the simultaneous occurrence of thyroid hyperplasia and exophthalmos in trout fry following resorption of the yolk sac. Microscopy of the hyperplastic thyroid revealed follicles lined by tall columnar epithelium, and the lumina were devoid of stainable colloid. Addition of sodium iodide to the water prevented the appearance of both the thyroid hyperplasia and exophthalmos. The observations suggest the occurrence of diffuse toxic goiter (thyroid hyperplasia), at least partly based on iodine deficiency, during early postembryonic development.

Nigrelli (1952b) described three thyroid adenocarcinomas occurring in a single specimen of each of the following: *Fundulus heteroclitus* (common killifish), *Cyprinodon variegatus* (sheepshead minnow), and *Angelichthys isabelita* (blue angelfish). The tumors in the killifish and sheepshead minnow were classified as malignant interacinous adenomas, a designation essentially equivalent to adenocarcinoma. All three tumors were characterized by proliferation of follicles, sometimes without lumens or colloid. There was also proliferation of interfollicular cells, infiltration and destruction of gill tissue, cartilage, bone, and muscle in the pharyngeal region, and invasion of the adventitia of larger blood vessels. No metastases were observed.

Baker *et al.* (1955) frequently observed thyroid hyperplasia in certain inbred strains of platyfish, *Xiphophorus maculatus*. In these fish, thyroidal tissue, both *normal* and *hyperplastic*, may be widely distributed: anteriorly as far as the choroidal gland, and posteriorly to the heart, spleen, intestine, and kidney. Moreover, hyperplastic tissue in the kidney displays papillary, cystic, and follicular patterns, is locally destructive, and may invade and perforate the body wall. Such behavior would seem to indicate that, in these instances, the thyroid tissue is adenocarcinomatous and that visceral metastases may have occurred. The authors suggest the additional possibility that the thyroid tissue in the kidney and at other sites may arise by differentiation *in situ* from unidentified precursor cells.

Stolk (1955b) induced hyperplasia of the thyroid gland in *Xiphophorus helleri* and *Lebistes reticulatus* by adding thiouracil to the aquarium water. The numbers of follicles and epithelial cells were increased. Follicular epithelial cells were tall columnar, and colloid was decreased to absent. In 15 fishes (6 platyfish and 9 guppies), irregular solid masses of trabecular cells formed and infiltrated surrounding organs, including gill apparatus, cartilage, *blood vessels*, and muscles. The author referred to these as hyperplastic adenomas.

Stolk (1956b, 1956d) also described infiltrative thyroid tumors in the cyprinid *Tanichthys albonubes* and the cichlid *Cichlasoma biocellatum.* Both of the tumors contained follicular and afollicular areas, and infiltrated adjacent structures, such as connective tissue, bone, cartilage, muscles, gills, and skin.

A recent and thorough review of thyroid tumors in teleosts is that of MacIntyre (1960). This article discusses the genetic and environmental basis for the appearance of thyroid tumors in teleosts. In addition, several cases of spontaneous hyperplasia, which regressed after addition of iodide to the water, are described in *Girardinus falcatus.* An extensively infiltrative growth in *Xiphophorus variatus variatus* is also described; this may have been an adenocarcinoma.

Woodhead and Ellett (1967) described tumorous enlargements (hyperplasia?) of the thyroid of senile guppies, *Lebistes reticulatus.* The thyroid of aging guppies is generally composed of inactive follicles with flat epithelium. However, in the two instances described, the follicular epithelial cells in the center of the gland became enlarged and swollen, and the enclosed colloid was coarsely granular. The appearance of some cells corresponded to that seen when fish are experimentally treated with thyrotrophic hormone over a long period. It was suggested that the thyroid hyperplasia in the guppy developed as a result of the inability of the aging gland to respond to thyrotrophic hormone.

The cases given illustrate the difficulty experienced in distinguishing between hyperplasia and neoplasia of the thyroid gland in fishes. The same difficulties are experienced in evaluating possible neoplasms of the thyroid gland in other vertebrates.

A-12. Pituitary

There are four descriptions of pituitary tumors in the bony fishes (table 14). Stolk (1953b) described a chromophobe adenoma in a guppy, *Lebistes reticulatus.* The fish had the appearance of an intersex with the female body form and the male color pattern. The anterior lobe of the pituitary gland was greatly enlarged and contained an adenoma composed of groups and strands of cells with finely granular, light blue (azan-staining) cytoplasm, and somewhat pleomorphic nuclei.

TABLE 14.—Osteichthyes: Epithelial tumors of the pituitary gland

Reference	Species	Number of fish	Pathology, comments
Stolk, 1953b	*Lebistes reticulatus* (guppy)	1	Chromophobe adenoma.
Stolk, 1958a	*Mollienesia velifera*	6	Chromophobe adenoma.
Stolk, 1958b, 1958c	*M. velifera*		Chromophobe adenoma.
Sathyanesan, 1962	*Mystus seenghala*	1	Basophilic adenoma (?)

Stolk (1958d, 1958g) also observed similar chromophobe adenomas in 6 of 84 mollies, *Mollienesia velifera.* Again, the fish were complex intersexes with the male color pattern and the female form, indicating endocrine alterations possibly related to the tumors. The cells were arranged in alveoli or strands, nuclei were pleomorphic, and the optic chiasma was displaced anteriorly.

Sathyanesan (1962) has reported a basophilic tumor (adenoma?) in one adult male *Mystus seenghala.*

A-13. Swim Bladder

There is a single instance of a tumor of the swim bladder, an adenoma in an adult female guppy. *Lebistes reticulatus* (Stolk, 1957h). The tumor was large, occupying most of the coelomic cavity and displacing the heart, kidneys, ovaries, intestine, liver, and spleen. Microscopically, there were glandular elements composed of light- and dark-staining epithelial cells supported in a vascular connective tissue stroma.

Mesenchymal Neoplasms in Osteichthyes

Most of the earlier references to mesenchymal tumors in fishes were previously reviewed by Schlumberger and Lucké (1948). Since many of the older reports are incomplete and of little value to the contemporary investigator, some have been omitted. For the most part, this review includes only those reports in which the species was given, and in which the description was reasonably diagnostic of a specific neoplastic entity.

In the text that follows, the mesenchymal tumors are grouped according to tissue of origin as follows: fibrous tissue (fibroma and fibrosarcoma), adipose tissue (lipoma), myxomatous tissue (myxoma and myxosarcoma), cartilage (chondroma), bone (osteoma and osteosarcoma), smooth muscle (leiomyoma), striated muscle (rhabdomyoma and rhabdomyosarcoma), blood vessels (hemangioma and hemangioendothelioma), lymphatic tissue (lymphosarcoma), notochord (chordoma), and sarcoma (anaplastic, mixed, or unidentified). Many examples of "spindle cell sarcoma" are grouped with the fibrosarcomas, since fibroblastic origin seemed most likely. Similarly, when descriptions of "round cell sarcoma" are suggestive of lymphosarcoma, the tumors are so classified. In instances where there is appreciable uncertainty about the diagnosis, this is indicated by appropriate placement of question marks. Note that malignant examples were not located for some tissues.

B-1. Fibrous Tissue: Fibroma and Fibrosarcoma

In the bony fishes the most common mesenchymal neoplasms originate from fibroblastic elements, giving rise to fibromas and fibrosarcomas (table 15).

TABLE 15.—Osteichthyes: Fibroma and fibrosarcoma

Reference	Species	Number of fish	Pathology, comments
Crisp, 1854	*Cyprinus carpio* (carp)	1	Fibroma, abdominal cavity.
Gervais, 1876	*C. carpio*	1	Fibroma, abdominal cavity.
Eberth, 1878	*Salmo lacustris* (lake trout)	1	Fibroma or fibrosarcoma (?).
Bland-Sutton, 1885	*Gadus morhua* (codfish)	1	Fibroma, abdominal wall.
	G. morhua	2	Fibroma, stomach.
	Carassius auratus (goldfish)	1	Spindle cell sarcoma (fibrosarcoma?), dorsal fin.
Plehn, 1906	*Carassius carassius* (Crucian carp)	1	Fibromas, multiple, abdominal cavity.
	Abramis brama (bream)	1	Fibroma, abdominal cavity.
	Esox lucius (pike)	1	Lipofibroma, trunk.
	C. carassius	1	Fibrosarcoma (?), testis.
	Chondrostoma nasus ("le Nez")	1	Fibrosarcoma (?), back.
Schroeders, 1908	*Scorpaena porcus*	1	Spindle cell sarcoma (fibrosarcoma?), tail muscles, with metastases to heart and liver.
Fiebiger, 1909a	*Pollachius virens* (codfish)	1	Fibroma, buccal cavity.
Guglianetti, 1910	*C. auratus*	1	Fibroma, retro-orbital.
Johnstone, 1911	*Arengus pilchardus* (sardine)	1	Fibroma, abdominal cavity.
	Melanogrammus aeglifinus (haddock)	1	Fibroma, operculum.
Drew, 1912	*Pleuronectes platessa* (plaice)	1	Fibrosarcoma or fibroma (?), operculum, pigmented side.
Fiebiger, 1912	*P. virens*	1	Fibrosarcoma, subcutaneous.
Johnstone, 1912	*G. morhua*	1	Fibrosarcoma, snout.
Wolff, 1912	*Anguilla anguilla* (eel)	1	Fibrosarcoma, mesentery.
Johnstone, 1913	*Hippoglossus hippoglossus* (halibut)	1	Fibroma, retroperitoneum.
Johnstone, 1914	*H. hippoglossus*	1	Fibromas, multiple, trunk muscles.
	G. morhua	1	Fibroma, left orbit.
Ronca, 1914	*C. carpio*	1	Fibroma, mesentery.
Johnstone, 1915	*G. morhua*	1	Fibrosarcoma (?), trunk.
Beatti, 1916	*Pogonias chromis* (croaker)	1	Fibromas or fibrosarcomas(?), subcutaneous, multiple.
Johnstone, 1922	*P. platessa*	1	Fibroma, dorsal fin, pigmented side.
Kazama, 1922	*Oncorhynchus keta* (dog salmon)	1	Spindle cell sarcoma (fibrosarcoma?), body wall.
Schamberg and Lucké, 1922	*C. auratus*	1	Spindle cell sarcoma (fibrosarcoma?), subcutaneous.
Wago, 1922	*C. auratus*	1	Myxofibroma, subcutaneous, multiple.

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Table 15.—Osteichthyes: Fibroma and fibrosarcoma—Continued

Reference	Species	Number of fish	Pathology, comments
Johnstone, 1923	*C. auratus*	1	Fibrosarcoma, caudal peduncle.
	Molva molva (ling)	1	Fibrosarcoma, head.
	Rhombus maximus (turbot)	1	Fibrosarcoma, trunk.
Johnstone, 1924	*M. aeglifinus*		Fibromas, subcutaneous, multiple.
	R. maximus	1	Fibroma, dorsal fin.
Roffo, 1924	*C. auratus*	7	Fibrosarcomas, subcutaneous. Involved 7 of 20 goldfish kept in a small aquarium.
Johnstone, 1925	*A. pilchardus*	1	Fibroma, peritoneum.
Leger, 1925	*Salmo gairdneri* (rainbow trout)	2	Fibrosarcoma (incomplete description).
Sagawa, 1925	*C. auratus*	1	Fibromas, subcutaneous, multiple.
Eguchi and Oota, 1926	*C. auratus*	1	Fibroma, posterior to head.
Johnstone, 1926	*H. hippoglossus*	1	Fibroma, head, pigmented side.
	P. platessa	1	Fibroma, head.
	P. virens	1	Fibrosarcoma, mandible.
	H. hippoglossus	1	Fibrosarcoma (?).
Roffo, 1926	*Micropogon opercularis* (croaker)	1	Fibrosarcoma (?), head and anterior trunk.
	M. opercularis	1	Fibrosarcoma (?), caudal peduncle.
Thomas, L., 1927a	*G. morhua*	1	Fibrosarcoma, subcutaneous, trunk.
	G. morhua	1	Fibrosarcoma, abdominal cavity.

Dominguez, 1928	*C. auratus*	1	Fibrosarcoma (?), subcutaneous, with possible metastases.
Freudenthal, 1928	*Carassius vulgaris* (carp)	1	Fibroma, ovary.
Takahashi, 1929	*O. keta*	2	Fibrosarcoma (?), body wall.
	Theragra chalcogramma (coal fish)	1	Fibrosarcoma (?), trunk.
	T. chalcogramma	1	Fibrosarcoma (?), occipital region.
	Lateolabrax japonicus (Japanese bass)	1	Fibrosarcoma (?), pharynx.
	Sillago japonica (Kisugo)	1	Fibrosarcoma (?), dorsum of neck.
	L. japonicus	1	Fibrosarcoma (?), multiple.
	T. chalcogramma	1	Fibroma, trunk muscles.
	Sebastodes inermis (rockfish)	1	Fibroma, body wall near caudal fin.
Montpellier and Dieuzeide, 1932	*C. auratus*	5	Fibrosarcoma, subcutaneous.
Smith, G. M., *et al.*, 1936	*Rasbora daniconius*	1	Fibroma, dorsal fin.
Kreyberg, 1937	*Salmo trutta* (brown trout)	1	Fibroma, abdominal cavity.
Lucké *et al.*, 1948	*C. auratus*	30	Fibromas and fibrosarcomas.
Stolk, 1954	*Xiphophorus maculatus*	1	Fibrosarcoma, head.
Stolk, 1957b	*Malapterus electricus*		Fibromas, dermis and subcutaneous, multiple.
Stolk, 1957c	*Lebistes reticulatus* (guppy)	1	Fibroma, heart.
Stolk, 1957e	*L. reticulatus*	1	Fibroma, intestine.
Walker, 1958, 1961	*Stizostedion vitreum*	Several	Fibromas and fibrosarcomas, dermal and subcutaneous. Virus-like particles by electron microscopy.
Milleman, 1968	*Oncorhynchus kisutch* (coho salmon)	1	Fibroma, back. (Dr. Milleman reported at least 40 coho salmon with this disease taken in July 1967 from the ocean off Newport, Oregon.)
Wellings, S. R., unpublished	*Hippoglossus stenolepis* (Pacific halibut)	1	Fibroma, trunk, left.
	H. stenolepis	1	Fibroma, mandible, left.

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There are several reports of multiple mesenchymal tumors, often diagnosed as fibromas or fibrosarcomas, in isolated goldfish (*Carassius auratus*) populations. Roffo (1924) found fibrosarcomas in 7 of a group of 20 goldfish kept in a small aquarium. Following removal of the diseased fish and disinfection of the tank, no further tumors developed. Similar neoplasms were noted by Montpellier and Dieuzeide (1932) in 5 goldfish confined together in the same aquarium. Lucké, Schlumberger, and Breedis (1948) observed 30 tumor-bearing goldfish, most of which were collected from three small ponds. These authors indicated that the disease was found in certain pools and not in others, and that once the disease became established in a particular pool, the incidence tended to gradually increase. The evidence so far implicates environmental etiological factors, such as infectious agents, rather than heredity. There is a likelihood that the tumors described in these reports were in reality neurilemmomas (*see* section on neural tumors).

Stolk (1954) described a fibrosarcoma located on the dorsal surface of the head of a platyfish *Xiphophorus maculatus*. The tumor was composed of fibroblasts and collagen and infiltrated the adjacent corium and muscle.

In one adult guppy, *Lebistes reticulatus*, a fibroma was observed originating from the left auricle of the atrium (Stolk, 1957c). The neoplastic tissue consisted of well-differentiated, fusiform fibroblasts and interlacing bundles of collagen. Blood vessels were abundant in the tumor. Stolk (1957e) also observed a fibroma of the intestine in a guppy. The neoplasm had caused a stricture and was well differentiated.

Multiple skin sarcomas were described on pike-perch, *Stizostedion vitreum*, by Walker (1958, 1961). The tumors apparently originated from the dermal connective tissue and the morphology varied from fibroma to fibrosarcoma. By electron microscopy, virus-like particles, approximately 1000 A in diameter, were present in abundance in the tumor cells. The morphology of the tumors and of the virus particles bore no resemblance to that of lymphocystis disease.

Fibromas and fibrosarcomas are rather commonly observed in salmon (Kazama, 1922; Takahashi, 1929; Milleman, 1968). A coho salmon, *Oncorhynchus kisutch*, with a fibroma of the anterior back was taken in July 1967 from the ocean off Newport, Oregon (fig. 6) (Milleman, 1968). The tumor measured about 10 × 6 cm and was white, firm, and covered by normally pigmented skin and scales. Histopathologically, the neoplasm was composed of well-differentiated collagenous tissue (fig. 7). Grossly similar tumors were observed on approximately 40 other fish of the same species during the same year. The relative roles of genetics and environmental etiological agents are as yet unclear, and the problem deserves further investigation.

Two Pacific halibut, *Hippoglossus stenolepis*, collected in Alaskan waters, each had one fibroma (Wellings, unpublished). In one instance, the tumor was located on the mandible, measured 7 × 8 × 6 cm, and was hard, gray-tan, and firm. In the other instance, the tumor was located on the trunk (fig. 8), measured 4 × 3 × 2 cm, and was exophytic and somewhat papillomatous in appearance. Microscopically, both fibromas were composed of mature connective tissue (fig. 9).

B-2. Adipose Tissue: Lipoma

Recently, a tumor that might best be designated a *fibrolipoma*, was observed on the caudal peduncle of a Pacific halibut *Hippoglossus stenolepis* (Wellings, unpublished). The tumor measured 2 × 2 × 2 cm and was roughly hemispherical, with a partially ulcerated surface (fig. 10). Microscopically, it was composed of lobules of mature fatty tissue, separated by prominent bands of mature collagenous tissue (fig. 11). Report of lipomas are summarized in table 16. There are no malignant examples.

TABLE 16.—Osteichthyes: Lipoma

Reference	Species	Number of fish	Pathology, comments
Bergman, 1921	*Esox lucius* (lake pike)	1	Lipoma, subcutaneous, trunk.
	Pleuronectes platessa (plaice)	1	Lipoma, subcutaneous, pigmented side.
Kazama, 1924	*Paralichthys olivaceus* (flounder)	1	Lipoma, trunk.
Takahashi, 1929	*Plecoglossus altivelis* (Japanese samlet)	1	Lipoma, subcutaneous, head.
Williams, 1929	*Hippoglossus hippoglossus* (halibut)	1	Lipoma, trunk.
	Callionymus lyra (dragonet)	1	Lipoma, subcutaneous, head.
Thomas, 1933c	*Pollachius carbonarius* (pollack coalfish)	1	Lipoma, liver.
Anadon, 1956	*Trachurus trachurus* (mackerel)	1	Lipoma, back.
Wellings, S. R., unpublished	*Hippoglossus stenolepis* (Pacific halibut)	1	Fibrolipoma, caudal peduncle.

B-3. Myxomatous Tissue; Myxoma and Myxosarcoma

Tumors composed of myxomatous tissue have been reported on several occasions, as indicated in table 17. Two of these were sufficiently infiltrative and atypical to warrant the diagnosis of myxosarcoma.

The most recent report is that of Stolk (1958a). A myxoma of the skin was described on the dorsal portion of the head of an adult male *Phenecogrammus interruptus*. The tumor was large, sessile, and measured 12 × 7 × 6 mm. The overlying skin was intact. Histologically, there were interlacing bundles of collagenous fibers which formed a network enmeshing stellate myxoma-type cells. The interstitial spaces were filled with mucoid material. The tumor was not encapsulated and was infiltrative.

B-4. Cartilage: Chondroma

There are six reports of chondromas in bony fishes; no new reports have been added since the review by Schlumberger and Lucké (1948). These cases are tabulated in table 18.

TABLE 17.—Osteichthyes: Myxoma and myxosarcoma

Reference	Species	Number of fish	Pathology, comments
Plehn, 1906	*Tinca vulgaris* (tench)	1	Myxosarcoma, subcutaneous, head.
MacIntosh, 1908	*Pleuronectes platessa* (plaice)	1	Myxoma, trunk.
Schroeders, 1908	*Psetta maeotica* (flounder).	1	Myxoma, subcutaneous, head.
	P. maeotica	1	Myxoma, subcutaneous, trunk and fins.
Wago, 1922	*Carassius auratus* (goldfish)	1	Fibromyxoma, skin and subcutaneous, multiple.
Johnstone, 1926	*Solea solea* (sole)	1	Myxoma, peritoneum.
Williams, 1929	*Rhombus maximus* (turbot)	1	Myxosarcoma, trunk.
Stolk, 1958a	*Phenecogrammus interruptus*	1	Myxoma, skin.

TABLE 18.—Osteichthyes: Chondroma

Reference	Species	Number of fish	Pathology, comments
Mulsow, 1915	*Carassius carassius* (crucian carp)	3	Chondromas, cranium.
Surbeck, 1917	*Barbus fluviatialis* (barbel)	1	Chondroma (osteochondroma?), mandible.
Surbeck, 1921	*Elops saurus* (sea pike)	1	Chondroma, subcutaneous.
André, 1927	*Phoxinus* (minnow)	1	Chondroma, mandible.
Thomas, L., 1932d	*Pollachius virens* (codfish)	1	Chondromas (2), preorbital, subcutaneous.
Nigrelli and Gordon, 1946	*Hemichromis bimaculatus* (jewel fish)	1	Osteochondroma, operculum.

B-5. Bones: Osteoma and Osteosarcoma

Osteomas and osteosarcomas have been reported a number of times (table 19). In several instances, there is some doubt as to the histological diagnosis and as to the malignancy of the lesion.

Although most of the reports are of isolated bony tumors, the common occurrence of multiple osteomas in the red tai, *Pagrosomas major*, is an unusual instance, as the osteomas are apparently observed only on the hemal spines of the sixth to eighth caudal vertebrae, are frequently multiple, and have not been observed in other species. The first reports were those of Kazama (1924) and Sagawa (1925). The largest series of 102 fish was described by Takahashi (1929), who thought that the tumors were related to prior fracture with the development of excess callus. Schlumberger and Lucké (1948) carefully examined three specimens by roentgenographic and histological means and found no convincing evidence of previous fracture. The problem represents a potentially fruitful area for the study of species, genetic, and environmental influences in the development of neoplasia.

TABLE 19.—Osteichthyes: Osteoma and osteosarcoma

Reference	Species	Number of fish	Pathology, comments
Bell, 1793	*Platax pinnatus* (butterfly fish)	Several	Osteomas, neural and hemal spines, multiple.
Wahlgren, 1876	*Esox lucius* (pike)	1	Osteosarcoma (?), anal fin.
Gervais, 1875	*Lepidopus* sp. (scabbard fish)	1	Osteomas, dorsal fin rays, multiple.
	P. pinnatus	1	Osteoma, fin ray.
Bland-Sutton, 1885	*E. lucius*	1	Osteoma, dental plate.
	Gadus morhua (codfish)	1	Osteoma, maxilla.
	G. morhua	1	Osteochondroma (?), vertebra.
Plehn, 1906	*E. lucius*	1	Osteoma, vertebra.
Schroeders, 1908	*Psetta maeotica*	1	Osteomas, vertebral spines, multiple (15).
Fiebiger, 1909b	*Cyprinus carpio*	1	Osteochondroma, cranium.
Williamson, 1913	*G. morhua*	1	Osteoma, vertebral body.
Beatti, 1916	*Pogonias chromis* (croaker)		Osteomas, vertebrae, multiple.
Kazama, 1924	*Pagrosomas major* (red tai)	6	Osteomas, hemal spines, 6th–8th caudal vertebrae, multiple.
Sagawa, 1925	*P. major*	2	Osteomas, hemal spines, multiple.
Takahashi, 1929	*P. major*	102	Osteomas, hemal spines, multiple.
	Paralichthodes olivaceus (flatfish)	1	Osteoma, spinous process, 3d lumbar vertebra.
	Theragra chalcogramma (? coalfish)	8	Exostosis (osteoma?, osteochondroma?), operculum and spines of dorsal fin.
	Sebastodes inermis (rockfish)	1	Osteoma, dorsal fin.
Williams, 1929	*G. morhua*	1	Osteoma, premaxilla, left.
	Pollachius virens (codfish)	1	Osteosarcoma, vertebrae, with invasion of muscle.
Thomas, L., 1932b	*Salvelinus fontinalis* (brook trout)	1	Osteoma (osteofibroma?), caudal fin rays.
	G. morhua	1	Osteosarcoma, trunk, posterior to pectoral fin.
Schlumberger and Lucké, 1948	*P. major*	3	Osteomas, hemal spines, 6th–8th caudal vertebrae, multiple.
	Alutera schoepfi (file fish)	1	Osteoma, 1st interneural spine.
	Chaetodipterus faber (spadefish)	1	Osteoma, pectoral girdle.
	C. faber	1	Osteoma vs. hyperostosis, 1st interhemal spine.
Sarkar and Dutta-Chaudhuri, 1958	*Wallago attu* (Indian catfish)	1	Osteogenic fibroma, premaxilla.
Wellings, S. R., unpublished	*Hippoglossus stenolepis* (Pacific halibut)	1	Lipo-osteoma, operculum, left.
	H. stenolepis	1	Fibro-osteoma, operculum, left.

Sarkar and Dutta-Chaudhuri (1958) described a hard, bony, lobulated tumor measuring several centimeters in greatest dimension on the premaxilla of an Indian catfish *Wallago attu*. Histologically, there were multiple spicules of bone growing in a fibrous matrix. The tissue was relatively well differentiated and was diagnosed as an osteogenic fibroma by the authors.

Two cases of bony tumor in the Pacific halibut, *Hippoglossus stenolepis*, have been recently observed (Wellings, unpublished). In both fish, the tumors were located on, and intimately fixed to, the operculum of the blind side. In one fish (fig. 12), the tumor measured 6 × 4 × 2 cm and formed a creamy-white mass projecting above the surface of the operculum. The overlying skin was intact. On cut section the tumor was white and gritty. Microscopically, there were mature trabeculae of cancellous bone growing in a fibrofatty matrix (fig. 13). A suitable name for the tumor is lipo-osteoma.

A grossly similar lesion on the second halibut measured 1.5 × 1.5 × 1.0 cm and was composed of bony spicules in a fibrous matrix; a suitable diagnosis is fibro-osteoma.

B-6. Smooth Muscle: Leiomyoma

There are a few reports of mesenchymal tumors that appear to be of smooth-muscle origin occurring in bony fishes (table 20). The first three cases in the table were reviewed by Schlumberger and Lucké (1948), who thought that the lesions were more suggestive of hyperplasia than of neoplasia.

Schlumberger (1949) described what was interpreted as cutaneous leiomyomas in goldfish, *Carassius auratus*. Three–four percent of the goldfish in a 3-acre urban pond had single or multiple tumors composed of broad interlacing bundles of spindle cells. These cells gave the characteristic reaction of smooth muscle when stained by the van Gieson method or

TABLE 20.—Osteichthyes: Leiomyoma

Reference	Species	Number of fish	Pathology, comments
Plehn, 1906	*Salmo gairdneri* (rainbow trout)	1	Leiomyoma, stomach.
	Alburnus lucidus (shiner)	1	Leiomyoma, trunk muscles, probably arising from the smooth muscle of blood vessels.
Pesce, 1907	*Salmo carpio* (trout)	1	Fibroleiomyoma, stomach.
Thomas, L., 1933b	*Clupea harengus* (herring)	1	Leiomyoma, stomach.
Schlumberger, 1949	*Carassius auratus* (goldfish)	Many	Leiomyomas (?), cutaneous (*see* text). (These may be neurilemmomas—*see* section on neural tumors.)
Kubota, 1955	*S. gairdneri*	1	Leiomyoma, oral submucosa.
Sarkar *et al.*, 1955	*Mystus Mseenghala*	1	Leiomyoma, fins, multiple.

with trichrome. In at least one tumor there was palisading of nuclei but no nerve fibers were demonstrable with the Bodian stain. The author apparently re-interpreted these tumors as neurilemmomas in subsequent manuscripts (Schlumberger, 1952, 1957).

Mesenchymal tumors are common in goldfish, and have been variously diagnosed as fibromas, leiomyomas, neurilemmomas, and neurofibromas. That all of these types occur with fairly high frequency seems possible; it seems more likely, however, that many of the older descriptions of fibromas, fibrosarcomas, and leiomyomas were in fact of neurilemmomas (*see* section on neural tumors). It would appear to be appropriate to re-examine the entire question of cutaneous tumors of goldfish by use of modern techniques of histochemistry and electron microscopy. The question is an important one, since, as Schlumberger pointed out on several occasions, there is evidence for an infective biological agent in the genesis of the tumors. This, together with the genetic, species, and environmental background, should provide a fruitful area for research.

Kubota (1955) described a leiomyoma located on the lower jaw and in the oral cavity of a rainbow trout, *Salmo gairdneri*. The tumor measured 4.6 cm in greatest dimensions, was multilobulated, and appeared to develop in the soft tissue beween the bone and overlying epithelium. Histopathologically, there were bundles of well-differentiated smooth muscle fibers.

Sarkar *et al.* (1955) described multiple leiomyomas (23) occurring on the fins of an Indian catfish *Mystus seenghala* (Sykes). The tumors varied in size from 2 × 4 mm to 9 × 26 mm and were solid, ovoid, glossy, and cream-colored. Histopathologically, there were bundles of well-differentiated smooth-muscle cells that gave the appropriate reaction to the van Gieson method.

B-7. Striated Muscle: Rhabdomyoma and Rhabdomyosarcoma

Neoplasms arising from striped muscle cells are reported in table 21. Since the 1948 review of Schlumberger and Lucké, at least one new case has been added to the literature, that of Finkelstein and Danchenko-Ryzchkova (1965). These authors observed a rhabdomyoma in a perch, *Perca fluviatilis.*

B-8. Blood Vessels: Hemangioma and Hemangioendothelioma

There are at least eight reports of tumors originating from blood vessels (table 22). The most recent report is that of Stolk (1958b) who observed a hemangioma of the operculum in *Chilodus punctatus.*

B-9. Lymphatic Tissue: Lymphosarcoma

Lymphosarcomas have been reported on several occasions (table 23). There are also a number of reports of "round cell sarcomas," the gross and microscopic descriptions of which suggest that they were probably lymphosarcomas. These merely doubtful examples have been included in table 23, but qualified with a question mark.

Table 21.—Osteichthyes: Rhabdomyoma and rhabdomyosarcoma

Reference	Species	Number of fish	Pathology, comments
Adami, 1908	*Salmo clarkii*	1	Rhabdomyoma (?), rhabdomyosarcoma (?), trunk musculature.
Fiebiger, 1909c	*Pollachius virens* (coalfish)	1	Rhabdomyoma, visceral.
Bergman, 1921	*Osmerus eperlanus*	1	Rhabdomyoma, posterior to head.
Young, 1925	*Rhombus maximus* (turbot)	2	Rhabdomyoma, trunk musculature, nonpigmented side.
Kolmer, 1928	*Tinca tinca* (tench)	1	Rhabdomyoma, trunk musculature.
Ladreyt, 1930	*Labrus mixtus* (wrasse)	1	Rhabdomyosarcoma, trunk musculature, with ulceration and infiltration.
Williams, 1931	*Clupea harengus* (herring)	1	Rhabdomyoma, trunk musculature.
Thomas, L., 1932a	*Hippoglossus hippoglossus* (halibut)	1	Rhabdomyosarcoma, trunk musculature, with infiltration.
Finkelstein and Danchenko-Ryzchkova, 1965	*Perca fluviatilis* (perch)	1	Rhabdomyoma.

Table 22.—Osteichthyes: Hemangioma and Hemangioendothelioma

Reference	Species	Number of fish	Pathology, comments
Plehn, 1906	*Gasterosteus aculeatus* (stickleback)	3	Hemangioma (hemangiosarcoma?), subcutaneous tissue, head.
Murray, 1908	*Gadus morhua*	1	Capillary hemangioma, trunk.
Drew, 1912	*Conger conger* (conger eel)	1	Hemangioendothelioma.
	Trigla lineata (gurnard)	1	Capillary hemangioma, operculum.
Johnstone, 1915	*Pungitius pungitius* (stickleback)	1	Hemangioma, eye.
Johnstone, 1924	*Scomber scombrus* (mackerel)	1	Hemangioma, trunk musculature.
Johnstone, 1925	*Molva molva* (ling)	1	Hemangioma, trunk musculature.
	Pleuronectes platessa (plaice)	1	Hemangiomas, trunk musculature, multiple.
Johnstone, 1926	*Pollacius virens*	1	Hemangiomas, trunk musculature, multiple.
	Merluccius merluccius	1	Hemangioma, rectum.
Stolk, 1958b	*Chilodus punctatus*	1	Hemangioma, operculum.

TABLE 23.—Osteichthyes: Lymphosarcoma

Reference	Species	Number of fish	Pathology, comments
Ohlmacher, 1898	*Esox lucius* (pike)	1	Lymphosarcoma (?), disseminated.
Schroeders, 1908	*Psetta maeotica* (flounder)	1	Lymphosarcoma (?)
Johnstone, 1912	*Platichthys flesus* (plaice)	1	Lymphosarcoma, retro-orbital tissue, left.
Johnstone, 1924	*Clupea harengus* (herring)	1	Lymphosarcoma, kidney, with spread to liver.
Plehn, 1924	*Carassius auratus* (goldfish)	1	Lymphosarcoma, kidney, with spread to liver.
Johnstone, 1926	*C. harengus*	1	Lymphosarcoma, coelom.
Johnstone, 1927	*Rhombus maximus* (turbot)	1	Lymphosarcoma (?), disseminated.
Takahashi, 1929	*Oncorhynchus gorbuschka* (humpback salmon)	1	Lymphosarcoma (?), disseminated.
	Lepidoptrigla alata (gurnard)	1	Lymphosarcoma, trunk musculature.
Williams, 1931	*Conger conger* (conger eel)	1	Lymphosarcoma, kidney.
	Gadus morhua (codfish)	1	Lymphosarcoma (?), trunk musculature.
Haddow and Blake, 1933	*Salmo salar* (Atlantic salmon)	1	Lymphosarcoma, kidneys, bilateral.
Smith, G. M., *et al.*, 1936	*Rasbora lateristriata*	1	Lymphosarcoma, abdominal cavity, with extension to gill.
Nigrelli, 1947b	*E. lucius*	12	Lymphosarcoma, kidney, with dissemination to liver and spleen.
	Astyanax mexicanus (Mexican characin)	1	Lymphosarcoma, branchial cavity, with extension or metastasis to gills, heart, lever, pancreas, kidneys.
Ehlinger, 1963	*Salvelinus namaycush* (lake trout)	Several	Lymphosarcoma, kidney.
Mulcahy, 1963	*E. lucius*	13	Lymphosarcoma.
Mulcahy and O'Rourke, 1964	*E. lucius*	Many	Lymphosarcoma.
Honma and Hirosaki, 1966	*Oncorhynchus keta* (chum salmon)	1	Lymphosarcoma (?), kidney.
	O. keta	1	Lymphosarcoma.

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Lymphosarcoma appears to be a characteristic neoplasm among some species of the genus Esox. Nigrelli (1947b) observed lymphosarcomas of the kidney in 12 northern pike, *Esox lucius*. These fish had been kept in the same tank for several years, and all developed the disease and died within 1 year. The largest mass of tumor, and presumably the primary tumor, occupied and replaced the kidneys, and was composed of cellular, lymphoblastic tissue. There was disseminated disease involving liver, spleen, and retroperitoneum in each case. Schlumberger (1957) cites unpublished data of Ritchie which indicate that lymphosarcoma is also common in the Muskellunge, *Esox masquinongy*. Tumors occurred in about 10% of the fish from Lake Scugog, Ontario. Still more recent studies of Mulcahy (1963) and Mulcahy and O'Rourke (1964) indicate an incidence of lymphosarcoma in *Esox lucius* in Ireland of from 0.05/1000–2.60/1000.

Honma and Hirasaki (1966) described two cases of apparent lymphosarcoma in chum salmon *Oncorhynchus keta*, which had been raised in the Enoshima aquarium. In one specimen there was swelling of the head kidney due to massive infiltration of lymphocytes. In the other salmon there was a large tumor of the right posterior portion of the fish. The tumor was ulcerated and composed of small round cells that infiltrated the adjacent musculature and connective tissue.

B-10. Notochord: Chordoma

Levy (1962) reported the experimental production of tumor-like lesions of the notochord (chordomas?) in fish embryos of the species *Oryzias latipes* (Japanese medaka), *Anoptichthys jordane*, *Trichogaster tricopterus*, *Aequidens portalegrensis*, and *Carassius auratus* (goldfish). Embryos that were immersed in a solution of β-aminoproprionitrile developed tumorous overgrowths of the notochordal tissue. Histologically, there were either large mature physaliferous cells or a combination of these and proliferating fibroblasts. There were no metastases, and the author thought the tumors to be true benign neoplasms because they developed after removal of the causative agent (β-aminoproprionitrile), and they did not regress with time.

B-11. Sarcoma: Anaplastic, Mixed, or Unidentified

There are a number of reports in which the nature of the neoplasm is uncertain. Most of these were probably sarcomas; however, some could have been anaplastic carcinomas. The older reports, some of which are listed in table 24, are generally of limited value but are included for completeness.

The most interesting sarcoma of undifferentiated type is that described by Wessing (1959) and Wessing and von Bargen (1959), occurring in *Lebistes reticulatus* and *Pristella riddlei*. The neoplasm was apparently caused by a virus, since it was transmissible by cell-free filtrate to the following species: *Lebistes reticulatus*, *Brachydanio rerio*, *Hyphessobrycon flammeus*, and *Haplochromis multicolor*. The infectious particles were

TABLE 24.—Osteichthyes: Anaplastic, mixed, or unidentified sarcoma

Reference	Species	Number of fish	Pathology, comments
Bugion, 1875	*Phoxinus laevis* (minnow)	1	Anaplastic sarcoma, giant cell pattern.
Plehn, 1906	*Salvelinus fontinalis* (brook trout)	1	Anaplastic sarcoma, trunk musculature.
	Idus idus (chub)	1	Anaplastic sarcoma (or carcinoma?), trunk musculature
	P. laevis	1	Anaplastic neoplasm, trunk.
Plehn, 1909	*Hucho hucho* (Danubian trout)	1	Anaplastic sarcoma, spindle cell pattern, liver.
Johnstone, 1920	*Conger conger* (conger eel)	1	Mixed mesenchymal sarcoma, head.
Jahnel, 1939	*Xiphophorus helleri*	17	Anaplastic sarcoma, retrobulbar tissue.
Nishikawa, 1955	*Acanthogobius flavimanus*	1	Anaplastic sarcoma, spindle cell pattern.
Wessing, 1959, and Wessing and von Bargen, 1959	*Lebistes reticulatus* (guppy)	Many	Anaplastic mesenchymal sarcoma, involving kidneys, musculature, heart, intestinal tract, and testis.
	Pristella riddlei		Transmissible by cell-free filtrate.

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sedimented at 40,000–50,000 rpm and were rapidly inactivated by drying and by 50% glycerol. The tumors most frequently involved the kidneys, but could disseminate to the body cavities, heart, intestine, and testis.

Pigment Cell Neoplasms in Osteichthyes

Pigment cell tumors, including melanomas, erythrophoromas, guanophoromas, and xanthophoromas, have been described in at least 24 species of bony fishes and in several hybrids (table 25). Melanomas are the commonest of the pigment cell tumors, there being only a few reports of the other types. In most instances, the descriptions indicate that the pigment cell tumors of fishes are usually invasive and therefore to be considered "malignant" biologically.

The well-known melanomas of swordtail-platyfish hybrids (*Xiphophorus helleri* × *Xiphophorus maculatus*) were first observed by Haüssler (1928), and since have been extensively studied from the genetic point of view by Gordon (1948, 1951a, 1951b) and by others. A recent review by Anders (1967) is a valuable guide to the literature dealing with the genetics and biochemistry of the tumors.

Wild forms of platyfish possess several different and distinctive color patterns, due to the distribution of macromelanophores in the skin. There is evidence that each color pattern is determined by a single color gene, the action of which is subject to control by a group of modifying genes. In the tumor hybrids, as a result of the cross with the swordtail, the platyfish macromelanophore color gene is present, but without the full complement of modifier genes. The result is uncontrolled proliferation of macromelanophores, eventuating in melanomas. By means of various genetic maneuvers, including the backcrossing of the F_1 hybrids to either parent, it is possible to vary the dosage of modifier genes and, accordingly, to vary the severity and time of onset of the preneoplastic melanosis and the eventual melanoma.

Several recent studies, some of which are reviewed by Greenberg and Kopac (1963), have extended the experimental analysis of the swordtail-platyfish melanoma system by means of biochemical and cytochemical parameters (Greenberg, Kopac, and Gordon, 1956a, 1956b; Greenberg and Kopac, 1960; Humm and Humm, 1960, 1963; Humm and Sylvia, 1965; Anders, 1967).

In an interesting report, Proewig (1954) demonstrated that the growth rate of melanomas arising in platyfish-swordtail hybrids can be altered by changing the ionic strength of the water. When the conductivity of the water remained at 25,000 ohms or higher, tumor growth ceased completely. Optimum growth was obtained at 1500–2500 ohms. A change in surface tension of the water from 72 dynes/cm to 52 dynes/cm resulted in enhanced tumor growth. Anders (1967) indicated that a large number of environmental factors can act on the genome of the hybrids so as to lower the age of appearance and increase the rate of formation of tumors.

Although it is not the purpose of this review to treat experimental analyses in depth, it should be pointed out that the great value of the swordtail-platyfish hybrid system lies in the fact that the genetics of melanomagenesis in these fish has been extensively explored. Therefore, biochemical approaches can now be used to elucidate the mechanisms that control the growth, metabolism, and neoplastic transformation of the macromelanophores.

Ocular melanomas were described in *Xiphophorus maculatus*, *Xiphophorus pygmaeus*, and in 9 hybrids of *X. maculatus* and *X. helleri* by Levine and Gordon (1946). These tumors apparently arose in the choroid, then involved the retina, and finally perforated the eyeball to invade surrounding tissue. Gordon (1946) presented evidence to indicate that there was a genetic basis for these neoplasms.

Nigrelli, Jakowska, and Gordon (1950, 1951) reported a number of xanthoerythrophoromas in the head region of several genetically related platyfish-swordtail hybrids. The major cellular element of the tumors was the xanthoerythrophore, a cell which contains both red (erythropterin) and yellow (lutein and zeaxanthin) cytoplasmic pigments. In some samples, melanomas developed in the more posterior part of the same fish, and these occasionally invaded, destroyed, and replaced the tissue of the xanthoerythrophoroma.

Nigrelli and Jakowska (1953) noted a melanoma in the African lungfish, *Protopterus annectens*. The neoplasm apparently arose in the renal capsule and had invaded and destroyed much of the renal parenchyma. Microscopically, there was extensive invasion of the renal substance by melanocytes. This fish also had a tumor of the testis which the authors diagnosed as a spermatocytoma (seminoma).

Stolk (1953e) observed a melanoma of the skin in an adult cyprinid *Brachydanio rerio*. The lesion was located on the trunk behind the right pectoral fin, and was composed of large melanocytes in a delicate connective tissue stroma. There were both pigmented and unpigmented cells, with transitions between the two types. The neoplasm infiltrated the musculature, skeleton, kidneys, air bladder, intestine, liver, and spleen.

Stolk has also reported "species-specific" melanomas in 3 other fish. Infiltrating ocular melanomas were observed in five littermates of the species *Anoptichthys jordani* (Stolk, 1958e, 1958g). Skin melanomas were observed in six guppy-mollie hybrids (*Lebistes reticulatus* × *Mollienesia sphenops* (1958f, 1958g). These were nodular, lobulated masses composed of melanophores, melanocytes, melanophages, and stroma. A similar tumor in a guppy-mollie hybrid was reported by Ghadially and Gordon (1957). Six littermates of the black angelfish, *Pterophyllum scalare*, developed cutaneous melanomas which the author felt had originated through the transformation of cutaneous melanocytes to micromelanophores (Stolk, 1960a).

Table 25.—Osteichthyes: Pigment cell neoplasms

Reference	Species	Number of fish	Pathology, comments
Prince, 1892	*Melanogrammus aeglifinus* (haddock)	1	Melanoma, skin of body.
Schroeders, 1908	Blenny (species?)	1	Xanthophoroma, trunk.
Johnstone, 1915	*Hippoglossus hippoglossus* (halibut)	1	Melanomas, skin, multiple.
Bergman, 1921	*Zoarces viviparus*	1	Melanoma, skin.
Johnstone, 1925	*Pleuronectes platessa* (plaice)	1	Melanoma, skin, pigmented side.
Haüssler, 1928	*Xiphophorus maculatus* (platyfish) × Xiphophorus *helleri* (swordtail) hybrids	Several	Melanoma, skin (first report in swordtail × platyfish hybrids).
Ingleby, 1929	*Lophius piscatorius* (angler fish)	1	Melanoma, skin
Kosswig, 1929a, 1929b, 1931	*X. maculatus* × *X. helleri* hybrids	Several	Melanomas, skin.
Takahashi, 1929	*Theragra chalcogramma* (coalfish)	2	Melanomas, skin.
	Pneumatophorus colias (chub mackerel)	1	Melanoma, skin.
	Chelidonichthys kuma (gurnard)	1	Erythrophoroma, skin, with infiltration.
	Hexagrammos atakii (fat cod)	1	Guanophoroma.
Reed and Gordon, 1931	*X. maculatus* × *X. helleri* hybrids	Many	Melanomas.
Thomas, L., 1931b	*Gymnosarda alleterata* (tunny fish)	1	Erythrophoroma.
Haüssler, 1934	*X. maculatus* × *X. helleri* hybrids	5	Melanomas.
Smith, G. M., 1934	*Pleuronectes americanus* (flounder)	1	Erythrophoroma, skin, pigmented side. Infiltrative with apparent metastases.

Smith, G. M., *et al.*, 1936	*X. maculatus* × *X. helleri* hybrids	1	Erythrophoroma, skin of dorsal fin, invasive.
Dollfus *et al.*, 1938	*Epinephelus gigas* (grouper)	1	Melanoma, branchial region.
Nigrelli and Gordon, 1944	*Menidia beryllina peninsulae* (silverside)	1	Melanoma, skin of trunk.
Levine and Gordon, 1946	*Xiphophorus maculatus* (platyfish)	1	Melanoma, eye. Arose in choroid, invasive.
	Xiphophorus pygmaeus	1	Melanoma, eye.
	X. maculatus × *X. helleri* hybrids	9	Melanoma, eye.
Nigrelli *et al.*, 1951	*X. maculatus* × *X. helleri* hybrids	Several	Xantho-erythrophoroma. Melanoma.
Nigrelli and Jakowska, 1953	*Protopterus annectens* (African lungfish)	1	Melanoma, kidney.
Stolk, 1953e	*Brachydanio rerio*	1	Melanoma, trunk, with infiltration of muscle, skeleton, kidneys, air bladder, intestine, liver, and spleen.
Ghadially and Gordon, 1957	*Lebistes reticulatus* (guppy) × *Mollienesia sphenops* (black mollie) hybrids	1	Melanoma, head.
Stolk, 1958e, 1958g	*Anoptichthys jordani*	5	Melanoma, eye, infiltrative.
Stolk, 1958f, 1958g	*L. reticulatus* × *M. sphenops* hybrids	6	Melanoma, skin.
Stolk, 1959b	*Nothobranchius guentheri*	1	Erythrophoroma, skin, infiltrative and with metastases.
Stolk, 1959c	*Ctenobrycon spilurus*	6	Guanophoroma, skin.
Stolk, 1959d	*Rivulus xanthonotus*	7	Xanthophoroma.
Stolk, 1960a	*Pterophyllum scalare* (black angelfish)	6	Melanoma, skin.
MacIntyre and Baker-Cohen, 1961	*Xiphophorus variatus xiphidium* (spike-tailed platyfish)	1	Melanoma, skin.

Stolk (1959b, 1959c, 1959d) reported three cases of pigment cell tumors other than melanomas. An erythrophoroma was noted dorsally behind the operculum on an adult male *Nothobranchius guentheri* (1959b). The tumor consisted of nodules which microscopically were composed of fusiform cells with small cytoplasmic pigment granules ranging from vermilion to orange. The pigment granules were also observed free in the tissue and in the cytoplasm of phagocytes. There was infiltration of adjacent connective tissue and muscle, and additional tumor nodules (metastases?) were observed in the liver, kidney, and spleen.

Guanophoromas were observed in the characid *Ctenobrycon spilurus* (Stolk, 1959c). The tumors arose in the skin of the operculum and trunk of 6 adult fishes (4 males and 2 females), and were silvery-white on cut section. Microscopically, there were pleomorphic tumor cells embedded in connective tissue. Cells and interstitial spaces contained large numbers of guanine crystals. The latter were yellow white, needle shaped, and doubly refractile.

Stolk (1959d) has also reported xanthophoromas in 7 adult female cyprinodonts, *Rivalus xanthonotus*. The neoplasms consisted of two types of cells. There were numerous large epitheloid cells with vesicular nuclei and orange-yellow cytoplasmic granules, and scattered, single, multinucleated giant cells which also possessed the orange-yellow pigment in the cytoplasm. The tumors were apparently genetically related; there was no mention of invasion of surrounding tissue.

Neural Neoplasms in Osteichthyes

Tumors of the peripheral nervous system have been reported in fishes (table 26); there are as yet no reports of tumors of the central nervous system. The most interesting instances are those in which histopathologically characteristic tumors were observed in genetically related fishes, or in fishes from the same body of water.

Lucké (1942) reported single or multiple nerve sheath tumors in 0.5–1.0% of snappers, *Lutianidae*, collected off Tortugas. A total of 76 tumor-bearing fish were observed in the species *Lutianus griseus*, *Lutianus jocu*, and *Lutianus apodus*. Tumors were not found in other resident species nor were other tumors observed in the three species bearing nerve sheath tumors. The neoplasms were located along the larger subcutaneous nerve trunks and histologically resembled the neurilemmomas commonly observed in humans. Antoni type A (fibrocellular) and Antoni type B (loose, reticular) tissue formations, characteristic of neurilemmomas, were present histopathologically. G. A. Young and Olafson (1944) reported similar tumors in the viscera (pancreas and mesentery) of 25 inbred hatchery-raised brook trout, *Salvelinus fontinalis*.[2]

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[2] There is a question as to whether the tumors observed by these authors were not infectious granulomas rather than neurilemmomas. *See* Sniezko, 1961; and Wood, E. M., Yasutake, W. T., and Lehman, W. L.: J Infect Dis 97: 262–267, 1955. Although in doubt, the citation of Young and Olafson remains in table 26.

Table 26.—Osteichthyes: Neural neoplasms

Reference	Species	Number of fish	Pathology, comments
Thomas, L., 1927b	*Gadus morhua* (codfish)	1	Ganglioneuroma, trunk.
Takahashi, 1929	*Limanda yokohamae* (plaice)	1	Ganglioneuroma.
Thomas, L., 1932c	*Sparus auratus* (gilthead)	1	Neuroepithelioma, olfactory plate.
Haddow and Blake, 1933	*Lepidorhombus megastoma* (flounder)	1	Ganglioneuroma, spinal ganglion.
Picchi, 1933	*Carassius auratus* (goldfish)	1	Schwannoma, tail.
Lucké, 1942	*Lutianus griseus* (gray snapper) *Lutianus jocu* (dog snapper) *Lutianus apodus* (schoolmaster)	76 (Total, all species)	Neurilemmomas, peripheral nerves, multiple.
Young, G. A., and Olafson, 1944	*Salvelinus fontinalis* (brook trout)	25	Neurilemmomas, multiple.
Schlumberger, 1952	*C. auratus*	Many	Neurilemmomas, neurofibromas.
Finkelstein and Danchenko-Ryzchkova, 1965	*Perca fluviatilis*	1	"Neurinoma sarcomatosum" (malignant schwannoma, neurogenic sarcoma).
McArn and Wellings, 1967	*Lumpenus sagitta* (snake prickleback)	1	Neurofibroma, snout.
Wellings, S. R., unpublished	*Gadus macrocephalus* (codfish)	1	Neurofibroma, barbule.

Peripheral nerve tumors, apparently neurilemmomas and neurofibromas, were observed in about 10% of goldfish, *Carassius auratus*, in a pond near Cleveland, Ohio, by Schlumberger (1952, 1957). The tumors presented as multiple lumps beneath the skin or in the abdominal cavity. Some of the neoplasms had invasive characteristics and anaplasia sufficient to indicate malignancy. Schlumberger points out the difficulty of establishing the histogenesis of mesenchymal tumors, and believes it is very probable that most of the reports of fibrosarcoma in goldfish were in reality cases of neurilemmoma. It also seems likely that neurilemmomas in goldfish may have also been mistaken for cutaneous leiomyomas.

Despite much discussion, however, doubt still remains as to the histogenesis of the common mesenchymal tumors of goldfish. A recent ultrastructural study (Duncan and Harkin, 1968) concluded that the cell of origin of the goldfish tumors is not similar to the cell giving rise to human nerve sheath neoplasms.

In any case, the examples given above of "species-specific" nerve sheath tumors in fish are of considerable importance in that a strong genetic basis is implied for the development of these neoplasms. This brings to mind a similar human disease, multiple neurofibromatosis (von Recklinghausen's neurofibromatosis).

A neurofibroma, 1.5 cm in diameter, was located distally on the tip of the barbule of a codfish *Gadus macrocephalus* (Wellings, S. R., unpublished). Microscopically, the tumor was identical to the mammalian counterpart (fig. 14).

Teratoma in Osteichthyes

Stolk (1953a, 1959a) has reported ovarian teratomas in the guppy, *Lebistes reticulatus*, and in the platyfish, *Xiphophorus maculatus*. In one of a group of 1,484 guppies, an ovarian teratoma contained mature connective tissue, muscle, cartilage, bone, blood vessels, and nervous elements; in one of a group of 1,179 platyfish, an ovarian teratoma was composed of the same tissue, with the addition of intestinal and glandular elements. In both instances, the ovary and surrounding tissue were infested by a parasitic phycomycete, *Ichthyophonus hoferi*. The sperm chambers of the ovaries contained no spermatozoa, and the author hypothesized that a chemical agent produced by *Ichthyophonus* was responsible for stimulating parthenogenetic development in unfertilized oocytes, the latter giving rise to the teratomas.

A teratoma of the skin was described on the ventral body surface of one adult male guppy, *Lebistes reticulatus* (Stolk, 1955a). The tumor contained several mature tissues, including cartilage, bone, blood vessels, glands, nervous tissues, and meninges.

Hisaoka (1961) recently reported teratomas in the guppy, *Lebistes reticulatus*. Eighteen fish, divided equally between males and females, had tumors located on the ventral aspect of the abdomen between the pelvic and pectoral fins. The tumors contained striated muscle, nervous tissue, epidermis, cartilage, bone, connective tissue, adipose, blood vessels, and melanophores. A similar tumor was also described in one female swordtail, *Xiphophorus helleri* by Hisaoka (1963).

DISCUSSION

Several points of importance to the comparative pathologist are emphasized by the present review, and are briefly discussed below.

1) Theoretically, we would expect to find neoplasia in all animals possessing organized tissues (Coelenterata and above), and to find clonal phenomena at least analogous to neoplasia at the cellular level of organization (Protozoa and Porifera). There are as yet, however, *no indisputable recorded instances of neoplasia in echinoderms or prevertebrates, other than one case in Branchiostoma.* The failure to recognize spontaneous neoplasms among the echinoderms and prevertebrates could be the result of minimal effort spent in searching for such tumors. The failure to induce neoplasms experimentally in these groups is based on very few attempts and is therefore inconclusive.

2) *Neoplasia is, on the other hand, commonly observed among cartilaginous and bony fishes.* Tumors, both epithelial and mesenchymal have been found to affect most tissues, organs, and organ systems. Histopathologically, the neoplasms of fishes are similar to their amphibian, reptilian, mammalian, and avian counterparts. Invasion, metastasis, and biological "malignancy" are all present in fish tumors. As in the instance of higher vertebrates, the neoplastic cells show variable states of differentiation and retain to a degree the form and function of the cell type of origin. Thus, *the neoplasms of fishes appear to be both homologous and analogous to the neoplasms of other vertebrates.*

3) It is frequently hypothesized that the *genesis* of neoplasia in wild and domesticated populations of animals depends on the level of naturally occurring and human-generated carcinogens (physical, chemical, and biological), and on the variation in species-specific susceptibility to particular kinds and amounts of carcinogenic agents. The *incidence* of neoplasia in a particular species varies geographically and ecologically, largely as a result of the variability in distribution of different kinds and amounts of the responsible carcinogenic agents. Several examples of fish tumors that support this hypothesis were cited in the present review. Melanomas of platyfish-swordtail hybrids illustrate the classical interplay of genetic (species- and strain-specific) variables, the effects of which can be modulated by alterations in the physical and chemical properties of the aqueous environment. In the well-known hepatomas of rainbow trout, a potent chemical carcinogen acts on a susceptible genome, producing a high incidence of tumors. The mesenchymal sarcoma of *Lebistes* and *Pristella* described by Wessing (1959) and Wessing and von Bargen (1959) is an example of a fish tumor transmissible by a cell-free filtrate, and therefore

presumably involves the interaction of a viral agent with a susceptible genome. The common skin tumors of flounders vary in incidence at different collecting sites in Puget Sound, suggesting a prominent role for environmental and ecological variables. These examples indicate the *applicability of the so-called "multiple factor hypothesis" to tumorigenesis in fishes.*

4) Neoplasia is the result of the appearance of new clones of cells with morphological and physiological properties that are inheritable, capable of further change, and inappropriate to the developmental state of the affected organism. In fishes, as in higher vertebrates, neoplasms tend to duplicate (express) in some degree the differentiated state of the cells (or tissues) in which they originate. Therefore, *tumors would be expected to show only those kinds of morphological and functional differentiation that are part of the cellular and histological spectrum characteristic of the taxon in which the tumor exists.* Osteomas, accordingly, are observed in bony fishes and above, never at lower levels of organization where bone does not exist. From the point of view of geological time, a neoplasm would not be expected to show differentiation characteristic of tissues not yet evolved; *e.g.*, chondromas could not have appeared before the evolution of the first cartilaginous fishes in Ordovician and Silurian times, 450–500 million years ago. It is possible to list many other examples where paleontological and comparative anatomical data tend to limit the interval of geologic time in which a particular tissue and its neoplastic derivatives can exist.

5) The host's response is conditioned by organic evolution and varies from taxon to taxon on a temporal basis. The environmental agents are the result of, and change with, the geological, physical, and chemical evolution of our planet and solar system. The nature and amounts of carcinogenic agents are partly dependent on man's own biological and cultural evolution which determines his ability to introduce or remove chemical, physical, and biological tumorigenic agents from his environment. (The appearance of epidemics of hepatomas in hatchery-reared rainbow trout is an outstanding example of the human cultural influence in tumorigenesis.) *Thus, we expect host response and carcinogenic potential of the environment to vary in time and space in both the long and short run.*

6) *Neoplasms tend to increase in frequency with the age of the individual organism and of the population of organisms.* This means that cancers are generally not frequent in wild populations, since these populations are susceptible to predation and death at an early age before neoplasms develop. For example, the incidence of leukemia and mammary cancer in wild mice is much lower than when the same mice are maintained in cages, protected from some of the exigencies of the environment, and allowed to grow old. Similarly, the incidence of tumors in captive fish has been shown to increase with age and to exceed that in the corresponding wild populations. There are some exceptions to the rule that tumors are more frequent in older animals; one of these exceptions is that of the epidermal papillomas of flounders.

7) It seems to many that neoplasia is a property of all or most living systems and a consequence of the manner in which the form and function of these systems are coded and controlled. Since neoplasms may be more frequent among the "higher" forms, *e.g.*, insects, vertebrates, *it seems likely that increasing susceptibility to neoplasia accompanies, and may be a consequence of, the acquisition of multiple specialized tissues and organ systems.*

NOTE ADDED IN PROOF

The following two references describing skin tumors were located just prior to the submission of this manuscript. It was not possible to carefully review their content.

(*1*) Phillips, J. B.: A review of the rockfishes of California (Family Scorpaenidae). Cal Fish and Game Bull 104: 138, 1957.

The author gives a brief description of skin tumors (epidermal papillomas?) and aberrant coloration in a single specimen of *Sebastodes rubrivinctus.*

(*2*) Levings, C. D.: A comparison of the growth rates of the rock sole, *Lepidopsetta bilineata* Ayres, in the Northwest Pacific waters. Fish Res Bd Can Tech Rept No. 36, 1967.

The author indicates that approximately 10% of rock sole taken in the western Gulf of Alaska and in Bristol Bay were affected by tumorous skin growths (epidermal papillomas?).

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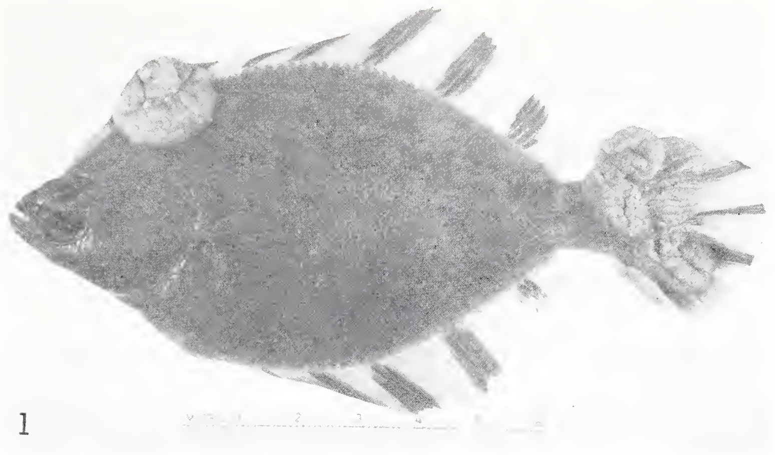

FIGURE 1.—Starry flounder, *Platichthys stellatus*, with two large epidermal papillomas, one located on the caudal fin, the other on the anterior dorsal fin and trunk.

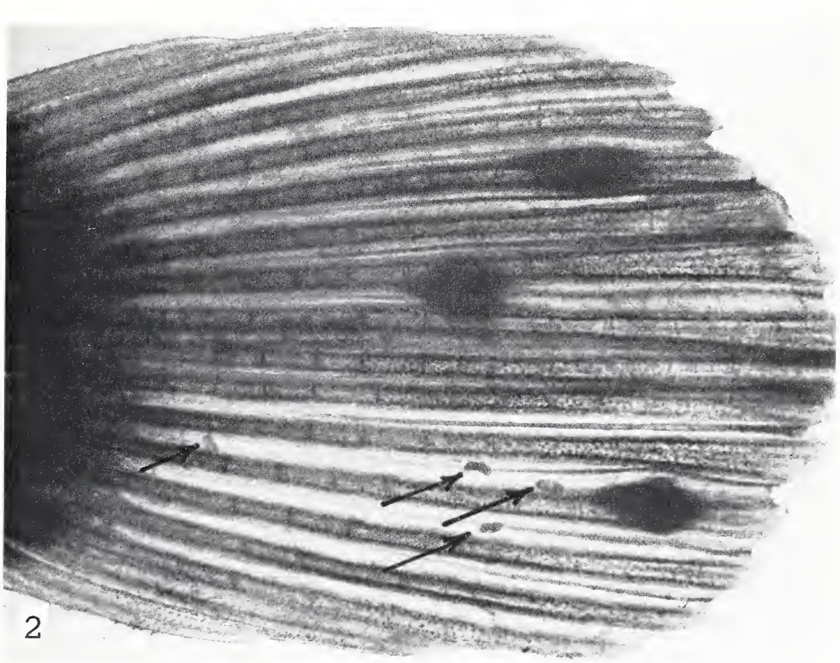

FIGURE 2.—Caudal fin of a starry flounder, *P. stellatus*, estimated age, 6 months. Overall length of the fin is approximately 7 mm. The three dark ovoid patches to *right* are "angioepithelial nodules," which are the precursors of epidermal papillomas. To *left* of *lower* nodule are three monogenetic trematodes of the genus *Gyrodactylus* (*arrows*) attached to the skin. A fourth is present at *far left*. These are common ectoparasites of the young fish and could conceivably play a role in tumorigenesis.

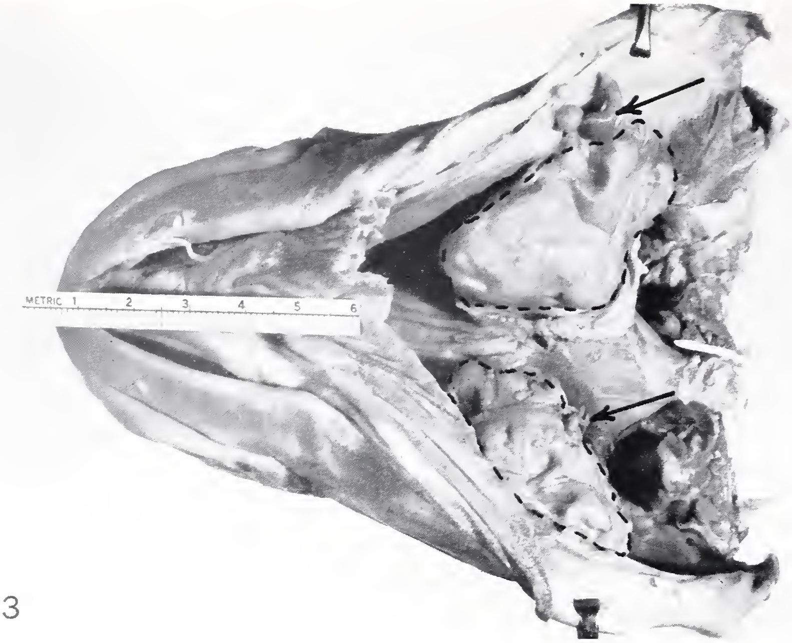

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FIGURE 3.—Ventral view of pharynx of codfish, *Gadus macrocephalus*. *Dotted lines* show the limits of two tumors interpreted as possible adenomas of the parabranchial bodies. *Upper arrow* indicates a parasitic leech, and *lower arrow* a parasitic copepod.

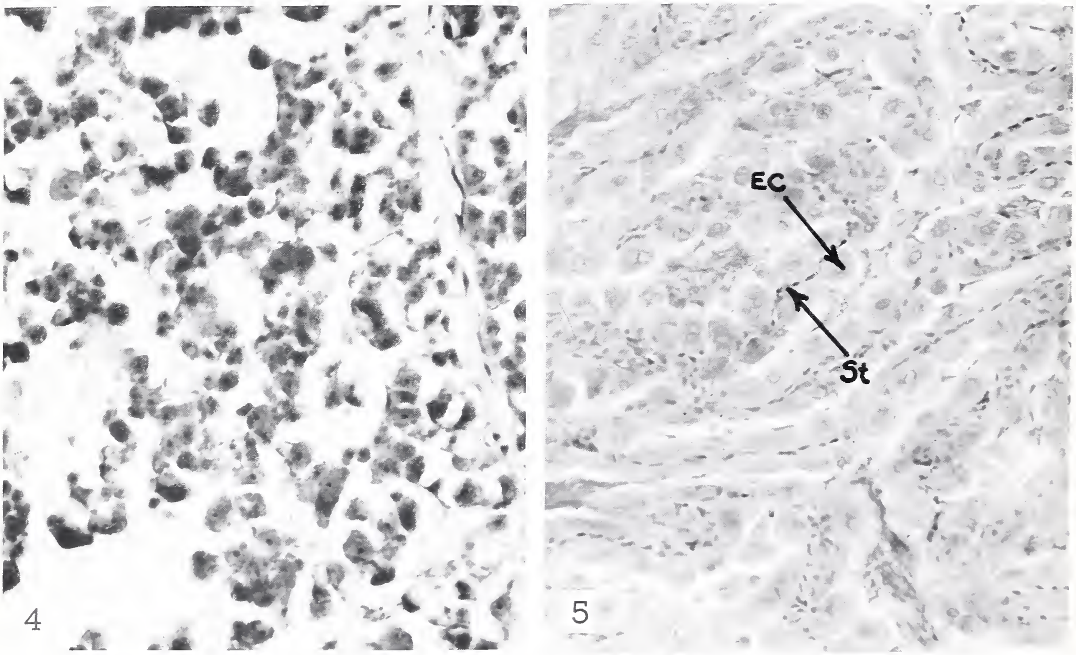

FIGURE 4.—Histopathology of tumor shown in figure 3. Loose aggregates of darkly staining cells with inconspicuous stroma. *Note* prominent nucleoli. Hematoxylin and eosin. × 470

FIGURE 5.—Tissue interpreted as residual parabranchial body, located in the interior of a presumed parabranchial adenoma. Stroma (St) is sparse and vascular. Characteristic epithelial cells (EC) of the parabranchial body are large with abundant eosinophilic cytoplasm. Epithelial cells, some of which are multinucleated, form a single layer of cells on the stromal support. Hematoxylin and eosin. × 270

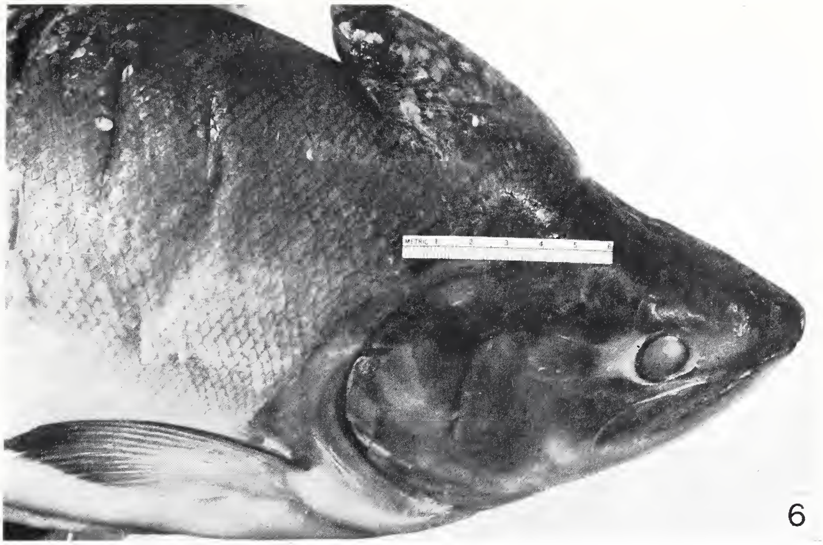
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FIGURE 6.—Fibroma, located on the back, just posterior to the head, of a coho salmon, *Oncorhynchus kisutch.*

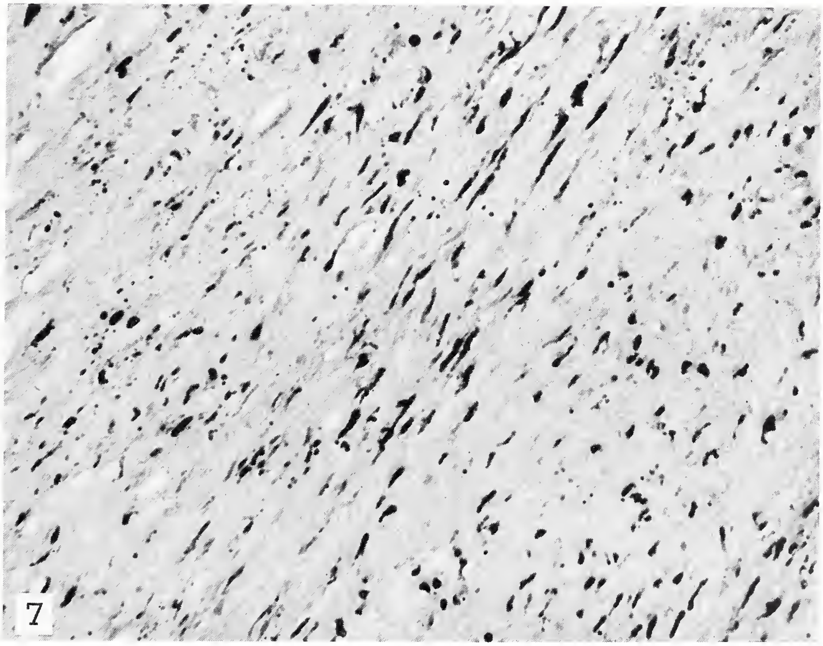
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FIGURE 7.—Fibroma of coho salmon (*see* gross, fig. 6). Parallel bundles of collagenous tissue and fibroblasts. Hematoxylin and eosin. × 470

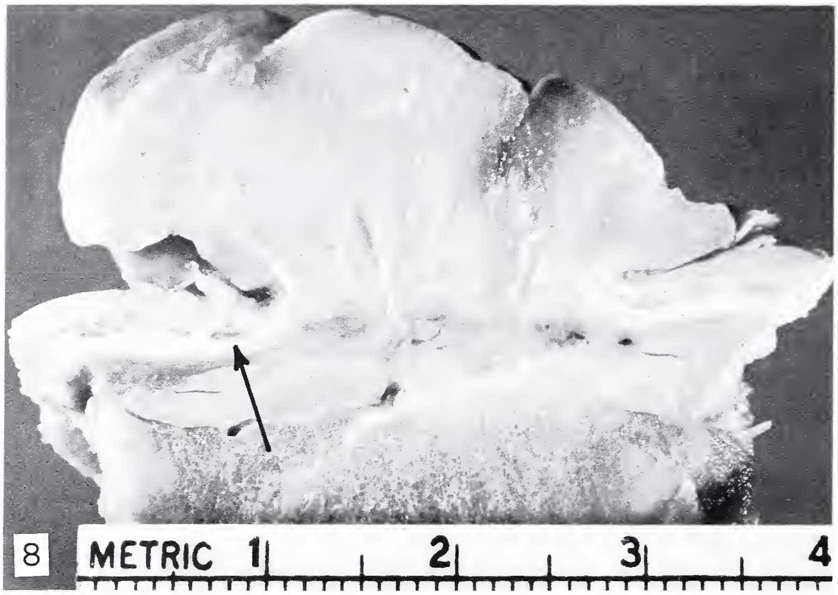


FIGURE 8.—Fibroma of trunk of a Pacific halibut, *Hippoglossus stenolepis*. *Note*, grossly, tumor appears to arise from the dense connective tissue of the dermis (*arrow*) and projects above the skin surface.

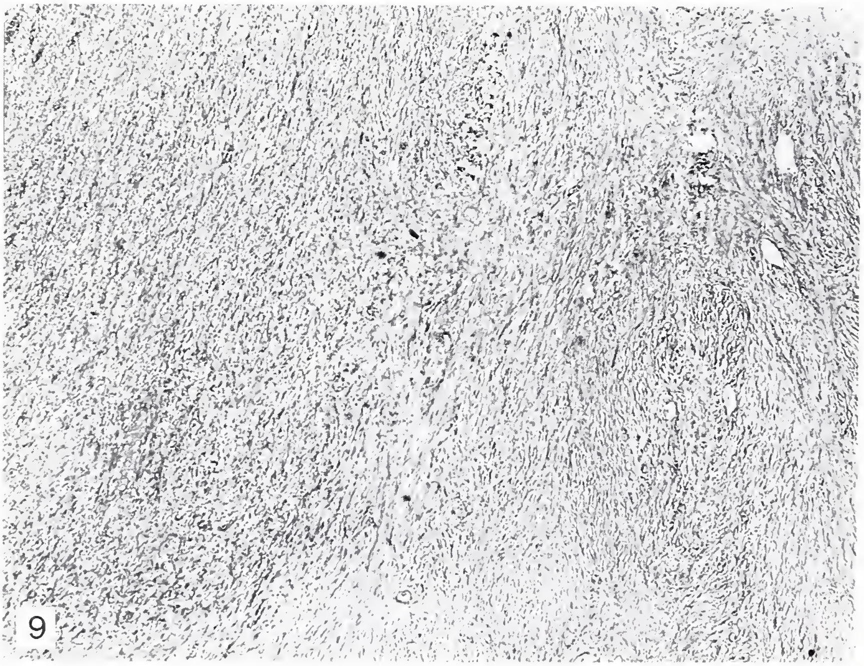


FIGURE 9.—Fibroma of the trunk of a Pacific halibut (*see* gross, fig. 8). Hematoxylin and eosin. × 34

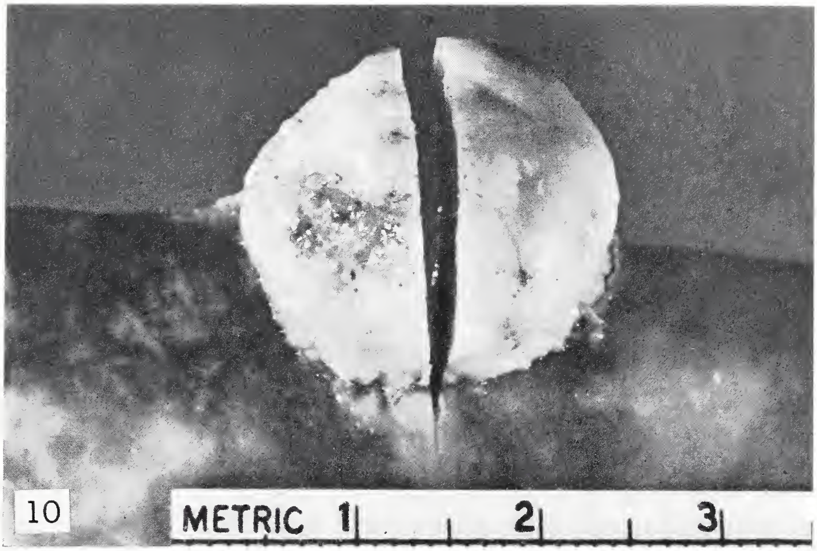


FIGURE 10.—Fibrolipoma of the caudal peduncle of a Pacific halibut, *Hippoglossus stenolepis*.

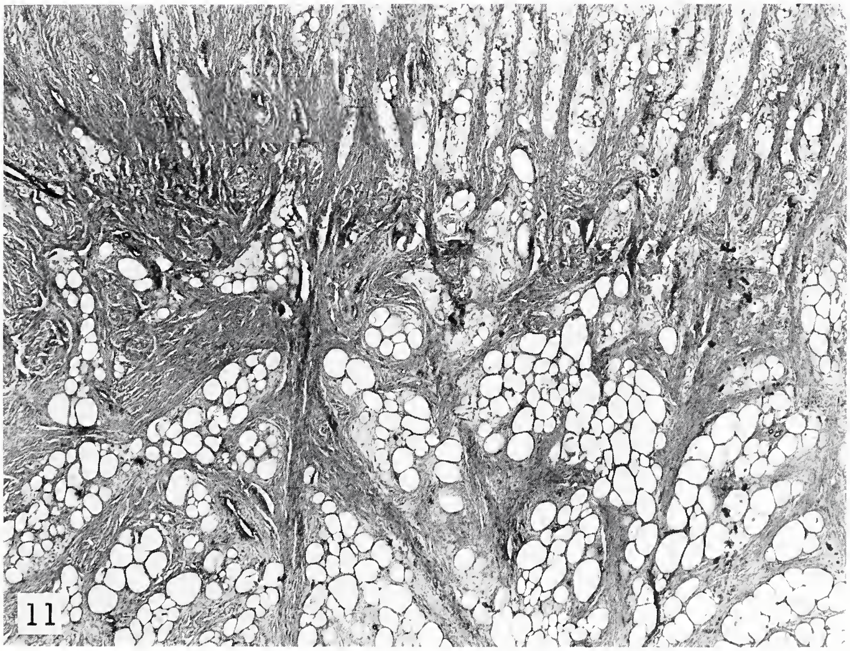


FIGURE 11.—Fibrolipoma of a Pacific halibut (*see* gross illustration, fig. 10). Islands of mature adipose tissue are enclosed by bands of collagenous tissue. Hematoxylin and eosin. × 34

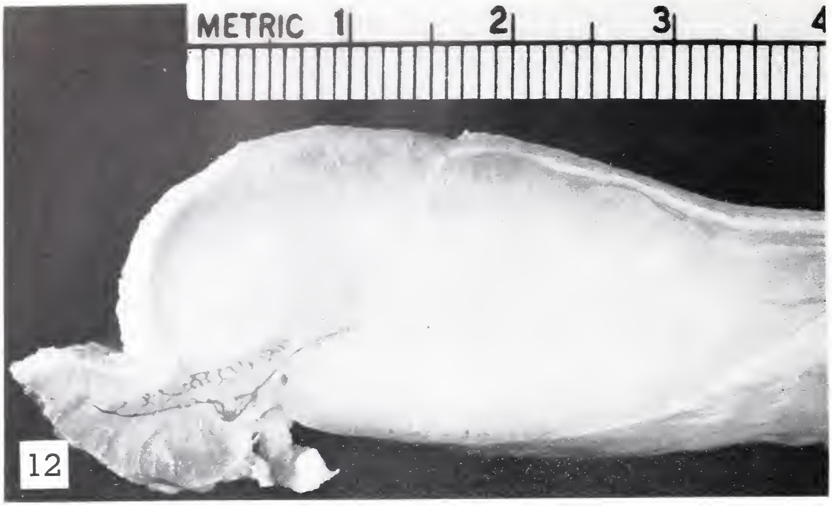
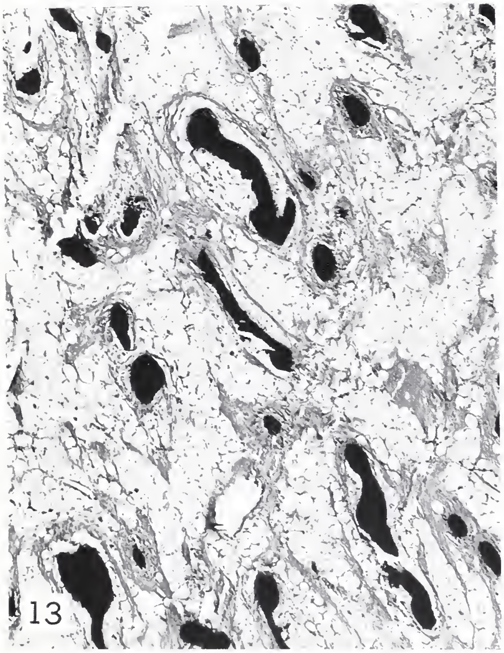


Figure 12.—Lipo-osteoma of the operculum of a Pacific halibut, *Hippoglossus stenolepis*. Tumor is the entire central, ovoid, pale mass of tissue.



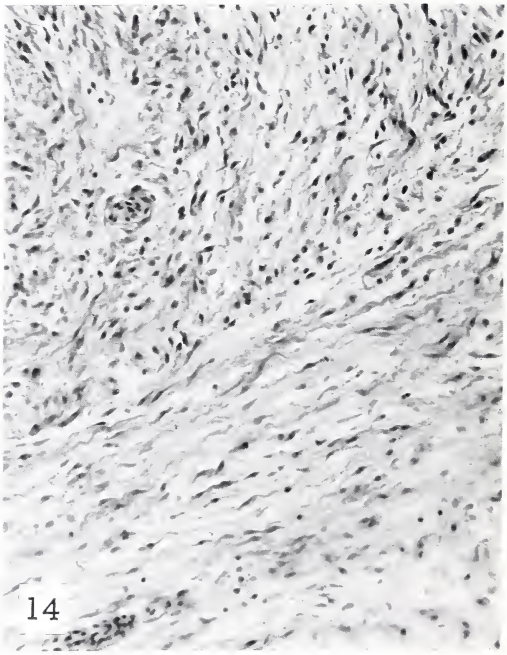


Figure 13.—Lipo-osteoma of the operculum of a Pacific halibut, *Hippoglossus stenolepis*. Darkest structures are trabeculae of cancellous bone. Interspersed between trabeculae is a continuum of fibrofatty tissue. Hematoxylin and eosin. × 34

Figure 14.—Neurofibroma of the barbule of a codfish, *Gadus macrocephalus*. Prominent microscopic features include wavy connective tissue fibers (oriented in parallel), a tendency toward palisading of nuclei, and the presence of nerve fibers in the tumor. Hematoxylin and eosin. × 270

Tumors and Aging

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SUMMARY

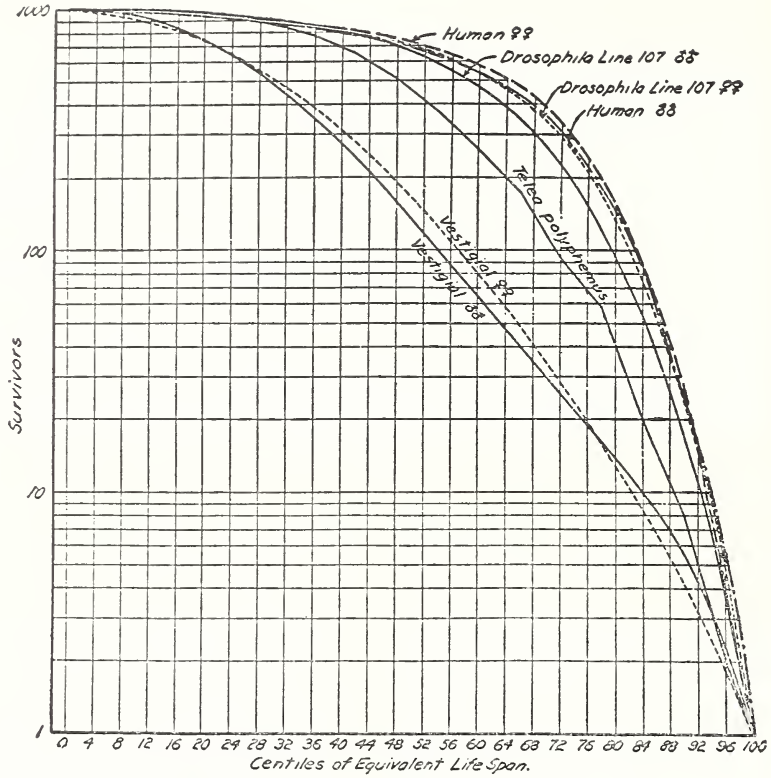
In higher vertebrates, including man, relationships between age and tumor development are well authenticated. In lower vertebrates and invertebrates, evidence is accumulating of similar relationships. Even in Protozoa, cellular changes occur with age. *Tokophrya infusionum* shows nuclear changes reminiscent of those seen in tumorigenesis. In the Coelenterata, cells change with age, individual cells can grow and multiply independently of the organism, and at least some evidence of tumor formation has been observed; but at present these findings have not been clearly correlated. In fish, on the other hand, there is considerable information on age-related tumors. Certain kinds of tumors, as in man, occur more often in young fish. Malignant tumors in general, however, are clearly more frequent in aged individuals.—Nat Cancer Inst Monogr 31: 129–140, 1969.

IN MAN and higher vertebrates in general, many well-authenticated relationships have been found between formation of tumors (benign and malignant) and the age of the organism. These include the greater frequency of certain types of tumors at an early age, the generally higher incidence of tumors in old age, and a number of more specific relationships related to age changes in individual organs.

Text-figure 1 compares survival curves of *Drosophila*, of the moth *Telea polyphemus*, and of man over the equivalent lifespans.

Rutgers (*1*) says (p. 165): "Variation in life-span between different inbred lines often is dependent on an inheritable predisposition to die of a particular cause, such as cancer or renal disease." He points out also that cancer is one of the causes of mortality which has an incidence curve that parallels the total curve of mortality.

Since neoplastic growth shows important relation to the aging process for the higher vertebrates, it is worthwhile to investigate a possible relationship for the lower vertebrates and even the invertebrates.



Text-figure 1.—Comparison of survival curves of *Drosophila*, of the moth *Telea polyphemus*, and of man. The lifespans of the 3 species have been made equivalent. The *Telea polyphemus* line shows both sexes together; the human and *Drosophila* curves are represented for male and female, with separate curves for the "vestigial" type of *Drosophila*. The general similarity of curve for man and fruit fly is seen.

Courtesy of Alfred A. Knopf Company. Pearl, R.: The Rate of Living, 1928, p. 185.

Tumorigenesis in relation to age in primitive vertebrates and invertebrates is a subject to which rather widely scattered observations must contribute. Such observations often are found in papers whose titles would not immediately direct the seeker to them for this type of information. Partly for this reason and partly because of limitations of space, my remarks on this subject will be confined to three groups of animals: Protozoa, Coelenterata, and Pisces. Hopefully, a further essay may cover other groups.

PROTOZOA

We might not anticipate that the Protozoa would offer us any interesting information on tumor development in relation to age. Tumor development, if it occurred, would be necessarily of a nature different from that in multicellular organisms; and the manner of reproduction might seem to preclude a definite aging process.

There are, however, certain one-celled animals, the suctorians, which reproduce by developing "embryos" within the parent body and releasing them by a process of delivery. Thus the parent protozoan might be considered as likely to undergo a process of senescence as would higher animals.

The remarkable finding made on the species *Tokophrya infusionum* (fig. 1) is that the macronucleus undergoes changes with age (*2*). In the young animal (2 or 3 days old), the macronucleus is about 5 μ in diameter and spheroidal. In the old animal (8 or 9 days) (figs. 2 and 3), it is 15–20 μ in diameter and is irregularly shaped, often developing many lobes. Also, there may be 2, 3, or 4 macronuclei in one "aged" cell. These macronuclei apparently arise by *hemixis*, a process described for Paramecium by Diller (*3*). In *Tokophrya*, hemixis is seen only in old cells. In a sense, this might be thought to be about as close as a unicellular organism can come to "tumor" formation.

Another remarkable change occurs in the internal organization of nuclei in old *Tokophrya* (*4*, *5*). The nuclear pattern, appearing originally homogeneous, changes (for that part of the nucleus between the chromatin bodies) to a regular pattern of light areas about 220 μ in diameter surrounded by denser material, giving a honeycomb effect (fig. 4). What relation, if any, this change has to nuclear lobation and division is unknown.

COELENTERATA

Crowell (*6*, *7*) reported some regressive changes in the hydranths of the colonial *Campanularia*. The young hydranth shows very little acid phosphatase activity. A "very old" hydranth of 10 days, however, has enormous (*6*, *7*) numbers of acid-phosphatase-containing granules. Crowell (*6*) and Strehler (*8*) measured changes with age in physiological capacity of *Campanularia*. Food-catching and digestion time did not seem to be altered. However, the "old" hydranths were only about 5 days of age, the maximum longevity of a considerable number of hydranths in Woods Hole at 17–18°C.

The level of adenosine triphosphate (ATP) in cells of old hydranths, however, was definitely decreased, as measured by Strehler's firefly ATP assay method.

From an early period, there has been much discussion in the literature as to whether coelenterates show a real process of aging. Pearl and Miner (*9*) constructed a life table for *Hydra*, similar to their table for *Drosophila*. David (*10*) kept detailed records of *Pelmatohydra oligactis* and concluded that they tend to die at an age of 20–28 months. Schlottke (*11*) challenged his conclusions because he felt that David's animals were heavily parasitized. Schlottke did find, however, *cellular changes* with age, primarily pyknotic alteration of nuclei of ectodermal cells, a removal of such cells into the endoderm, and the accumulation of guanine deposits, apparently from the remains of degenerating cells.

"Colonial" senescence, such as frequently occurs in protozoan cultures, apparently does not occur in coelenterates as a regular process, for lines of *Hydra* in which asexual budding was the only means of reproduction have been kept for decades (*12*).

Crowell (*13*) showed that aging in *Campanularia* markedly affects the growth rate of the uprights (stems with their hydranths) depending on nutritional conditions. In well-fed groups, young and old uprights grew at the same rate. When the nutritive level was decreased, the growth was inhibited in the young and to a greater extent in the old.

The ability of individual cells or groups of cells in any given organism to grow and multiply independently of the general economy of that organism, as in a tissue culture, may seem to indicate at least the capacity of these cells to form neoplasms. In *Hydra*, it has been shown recently (*14*) that such growth and multiplication of dissociated cells do occur under the proper conditions.

During studies on the mechanism of morphogenesis, Lesseps (*15*) cultured dissociated cells of Drosophila embryos, showing, as indeed has been demonstrated now for various arthropods, that the cells will grow and multiply in an environment largely independent of "organismal" control.

Evidence for the formation of neoplasia in Coelenterata has been presented recently (*16*). Studying the deep-water colonial coral *Madrepora kauaiensis*, Squires found evidence of peculiar anomalous growth. He described the three corallites considered to be neoplastic as having the following differences from normal ones: 1) total size, 2) arrangement of the septa, and 3) pattern of skeletal deposition. These three corallites were compared quantitatively with 50 randomly selected, normal ones of the 239 in this same colony.

In the three pathologic specimens, growth at unusual rate appeared to have occurred and at its greatest development this growth became chaotic and lost its relation to the normal symmetry of the polyp. Also the pathologic corallites appeared to arise from "normal" ones and in turn gave rise to normal ones. Squires (*16*) gives a number of reasons why the changes in these corals probably were not the result of injury or other environmental influence.

In view of the previous lack of evidence of neoplasia in coelenterates (*17*) and failure to induce neoplasia in one experimental study (*18*), these findings on *Madrepora* are of very great interest.

Thus in the coelenterates we do have *a*) some evidence of aging of individuals in certain species, *b*) cytological changes of degenerative nature with advancing age, *c*) demonstrable effects of age on the growth pattern, and *d*) some evidence of neoplastic growth in the corals.

Further observational and experimental studies are needed to determine whether true neoplastic growth occurs in this great phylum of invertebrates; but the evidence already on hand should be a considerable stimulus to such studies.

PISCES

Rearing fish in widely distributed hatcheries in the United States has made possible observations on the incidence of tumors in large populations. The rainbow trout (*Salmo gairdnerii*), for example, has been found to develop a type of primary liver cancer, hepatocarcinoma, which in some instances affects practically 100% of the population of fish over 3 years of age (*19*). This tumor is slow growing, and large tumor masses may be present in animals apparently still in general good condition. However, it metastasizes to kidney and spleen and may directly invade skeletal muscle.

The observations were based on examination of 143 rainbow trout ranging in age from 3–72 months (6 years). One group of fish from the National Aquarium had grossly normal livers, regardless of sex and age of the fish. Those from the Federal and State hatcheries, on the other hand, often displayed severe pathological lesions which *increased in frequency and severity with the age of the fish.* All fish below the age of 12 months had grossly normal livers. Earliest histological changes were small focal proliferations of slightly atypical, large, and more deeply staining liver cells.

Hepatic carcinoma in high incidence in artificially reared trout in the United States was first observed in April 1960 (*20*) when fish in transit from a commercial fish hatchery in Idaho were stopped at the California border. Trout with this condition often appear "normal" and grow rapidly. Indeed, many times they apparently will live for half or three-fourths of their normal lifespan. This high mortality in shipping seems to be due to the decrease in detoxifying capacity of the tumor-bearing liver.

The tumors vary from benign adenomas to completely anaplastic carcinomas. It is difficult to point to any "pretumorous" type of liver, although the early lesions usually are seen in fatty liver, and ceroid pigment often is present. Both fatty liver and ceroid are, however, common findings in livers of these fish. A black pigment, identified as melanin, also is a common finding.

Wood and Larson (*20*) point out that in a population where hepatic carcinoma was of high incidence, *gross lesions* were seen in approximately 50% of older fish (14–18 months old). In younger fish (1–4 months old), no gross lesions were observed, but *microscopic lesions* were detected in at least 25%. This finding was made in the Idaho hatchery mentioned above.

Wood and Larson believe that the etiologic agent may be a chemical or a hormonal carcinogen. They stress the point that apparently 100% of an animal population is involved by tumor formation.

The relation of hepatic carcinoma to age is seen in the consideration of its incidence as described here. Another and very important point is that in a population such as this, the lifespan of the species, as represented by this population, is determined to a considerable extent by this specific pathological process of tumor development.

Perhaps we could agree here with Montaigne who said: "Death is but an end to dying."

A review of the subject, *Hepatomas in Trout* (*21*, p. 209), attributes these tumors to rations containing cottonseed meal and cites particularly the work of Wolf and Jackson (*22*). It states (p. 209): "The larger the fish, the more advanced the disease." Some European investigators have tried to link these hepatomas with viral or other infectious agents. Very interestingly, Wolf and Jackson found that the presence of liver in the diet seemed to overcome the hepatogenic action of cottonseed meal. These authors point out that liver feeding also prevented the hepatogenic action of butter yellow in rats.

Rainbow trout reared in the hatchery have a tumor incidence which would seem to be clearly linked with age. In this case, fortunately, knowledge of the environment of the organism and the ability to control it experimentally have enabled us to assign an extrinsic cause to the development of the tumor. Yet the fact that tumors are not seen in younger fish may indicate a difference in susceptibility as much or more than a lack of time for action of the environmental influence.

In a small sample of the white sucker (*Catostomus commersoni*), a very high incidence of intrahepatic neoplasms was found in a recent study (*23*). The tumors were of intrahepatic bile duct origin. They were present in 3 of 12 specimens studied. All the fish were in the maximal size range of this species in Maryland. The authors say (p. 1196): "A probable source of bias that would favor a high incidence in our sample is the relatively old age of half the fish examined." They discuss several environmental factors which may play a role in etiology, but again the age relationship was a prominent one.

A reverse type of age relation seems to be true for the incidence of epidermal papillomas in the flathead sole, *Hippoglossoides elassodon* (*24*). In a total of 4,364 fish, 281 bore tumors, a 6.4% incidence. The tumors originated in the first year of life as angiomatous proliferations in the dermis, covered by a hyperplastic epidermis. From those, the epidermal papillomas developed, while the dermal component regressed. The number of tumors in all stages was highest in the first year and decreased with age. No tumors of these types were observed in flathead sole over 4 years of age. The tumor incidence was higher on the upper (right) side of the fish which is much more heavily pigmented.

Hisaoka (*25*) collected and described teratomatous growths in 18 specimens of guppy, *Lebistes reticulatus*. This appears to be the first description of teratoma in fish. The tumors consisted of a number of types of well-differentiated tissue. These tissues included bone, cartilage, nervous tissue, epidermis, adipose tissue, dense and areolar connective tissue, blood vessels, striated muscle, and thyroid follicles. Occurring in males and females with apparently equal frequency, the tumors were always located on the ventral body wall about midway between pectoral and pelvic fins. Movements of the fish seemed, in general, to be normal.

The fish were born with these tumors, and their lifespan was shortened. As the fish grew older, lordosis developed and large blood vessels led to the tumor. Thus the tumor seems to compete with the host. Hisaoka believes that teratomas in guppies are related to a twinning phenomenon. The tumors could be arranged as a graded series from an oval mass to an elongated body resembling a small headless fish. He was able to obtain 4 specimens of genuine "siamese" twins of this species, attached belly to belly.

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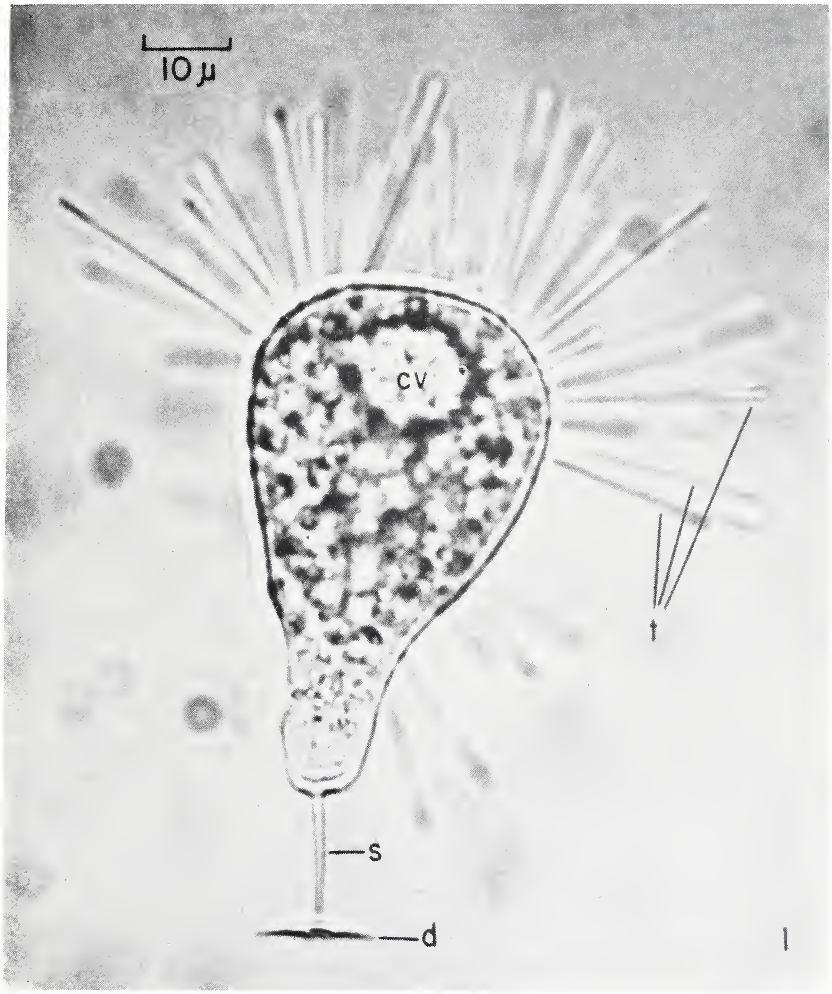

FIGURE 1.—Individual organism of *Tokophrya infusionum* showing contractile vacuole (CV) ; disc (d) ; stalk (s) ; and tentacles (t). × 1200

Courtesy of *Journal of Gerontology* (2).

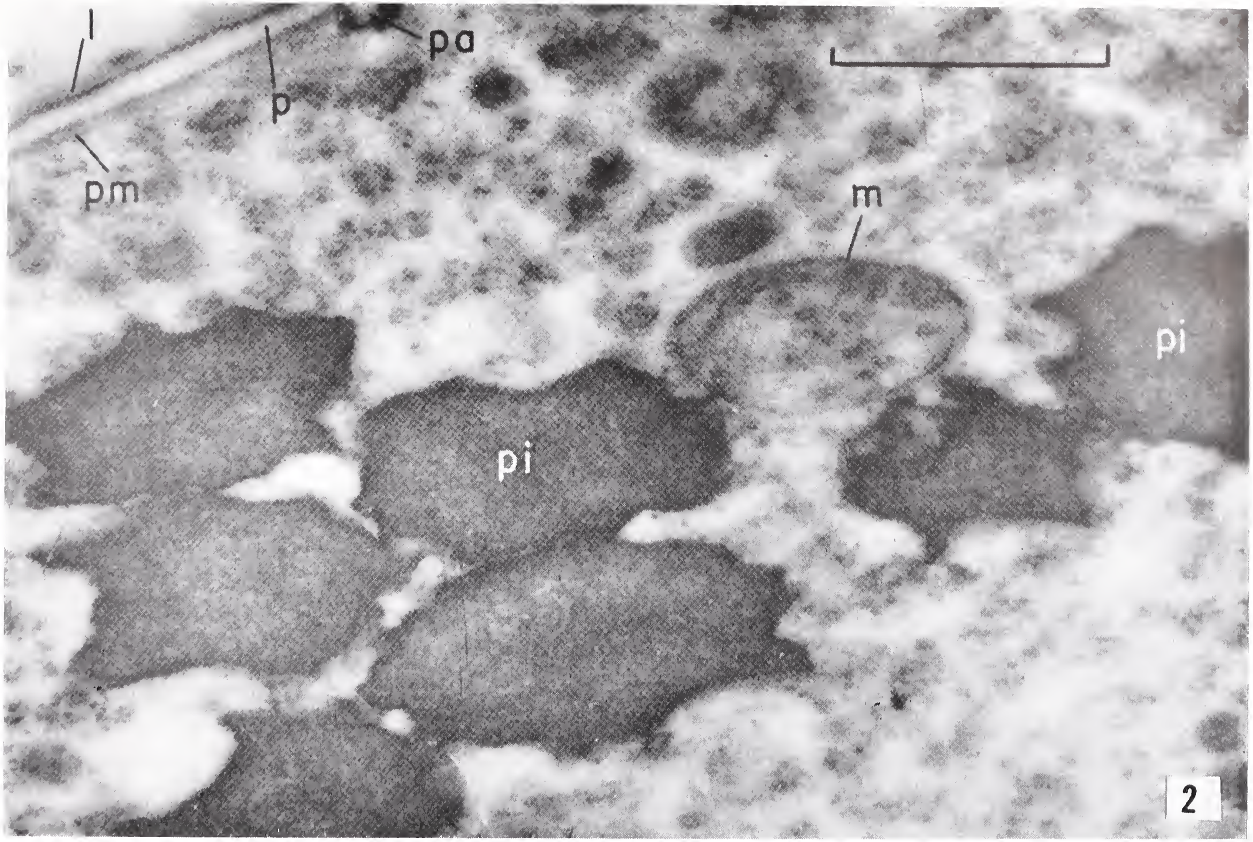

FIGURE 2.—Electron micrograph of cytoplasm of an old *Tokophrya infusionum*. Pigment granules are abundant; layer of homogeneous substance outside of pellicle (l); mitochondrion (m); pellicle (p); papilla (pa); pigment (pi); and plasma membrane (pm). × 31,000

Courtesy of *Journal of Gerontology* (2).

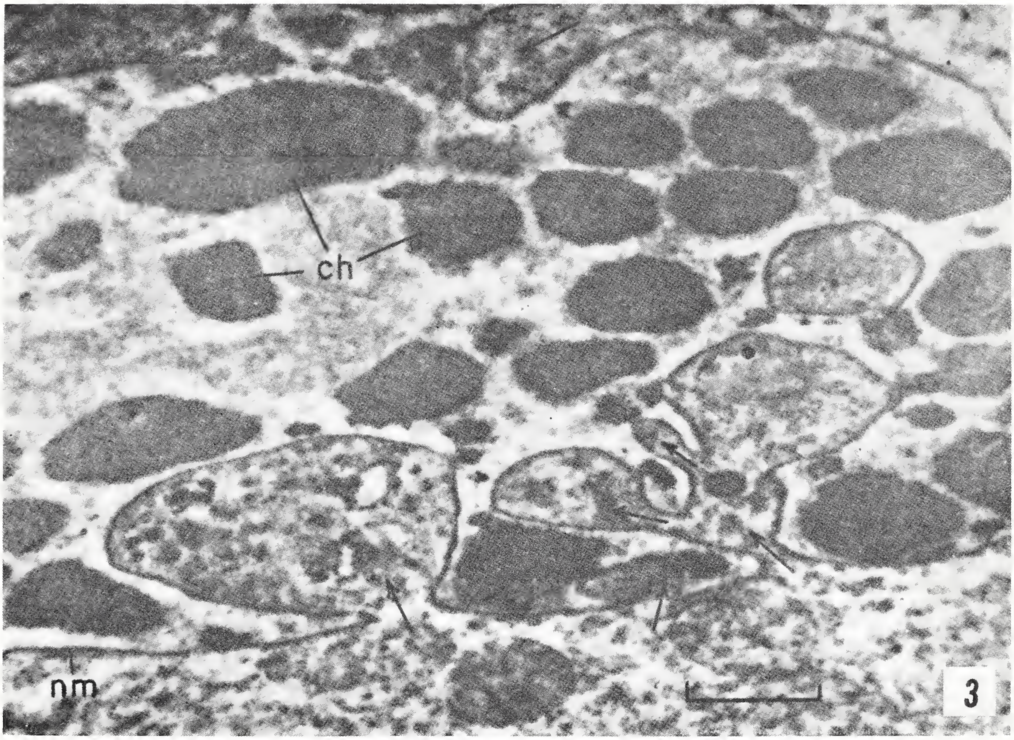

FIGURE 3.—Electron micrograph of macronucleus in old *Tokophrya infusionum* with deep invaginations (*arrows*) of the nuclear membrane (nm). The nucleus contains chromatin bodies (ch). × 17,700

Courtesy of *Journal of Gerontology* (*4*).

FIGURE 4.—Electron micrograph of longitudinal section of a chromatin body, showing a regular, crystal-like structure. In old *Tokophrya infusionum* a certain portion of the chromatin bodies commonly show such a pattern. × 67,000

Courtesy of *Journal of Gerontology* (4).

Aflatoxicosis in Coho Salmon

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SUMMARY

Young coho salmon, force-fed aflatoxin B_1 at 5, 10, or 15 mg toxin/kg body weight in 5 or 10 consecutive daily doses, developed toxic liver damage within 10 days post intubation when held in 15°C water. Acute hemorrhagic liver necrosis and subsequent mortality only occurred after ingestion of 8–10 mg of aflatoxin B_1/kg body weight over at least a 5-day period. Histology revealed areas of hepatocyte regenerative hyperplasia at each level of toxin tested. Rainbow trout of the same size given 0.5 mg aflatoxin B_1/kg body weight/day for 5 days all died by Day 11 and showed massive hemorrhagic liver necrosis, bloody ascites, and other physiological organ toxicosis. Comparative bioassay with trout and coho salmon fed aflatoxin B_1 at 20 μg/kg dry complete test diet daily for 20 months in 15°C water showed no tumors in salmon and typical hepatoma in all surviving trout. Coho salmon were 10–30 times more resistant to aflatoxicosis than rainbow trout and were generally refractory to chronic dietary treatment designed to induce hepatoma.—Nat Cancer Inst Monogr 31: 141–155, 1969.

THIS PAPER records acute aflatoxicosis in young coho salmon (*Oncorhynchus kisutch*) insulted by ingestion of crystalline aflatoxin B_1. Chronic effects from diets containing 1, 5, or 20 μg of aflatoxin B_1/kg total-diet dry ingredients did not occur in coho salmon fed a continuous daily insult of aflatoxin for 20 months, whereas hepatomas appeared in rainbow trout (*Salmo gairdnerii*) exposed to identical dietary treatments for the same time periods (*1*). The sensitivity of rainbow trout fingerlings to aflatoxin has been well documented (*2–5*) and recently reviewed (*6*). However, comparative sensitivity for both acute and chronic aflatoxicosis in coho salmon reared by the millions yearly in federal and state hatcheries to supplement and maintain the salmon fishery in the Pacific Northwest was not known and obviously needed to be tested.

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[1] We are very grateful to Dr. G. N. Wogan, Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Mass., who furnished the crystalline aflatoxin B_1 used in these experiments, and to Mr. Max E. Larson and Mrs. Penelope Turner of the Hagerman Field Station, Western Fish Nutrition Laboratory, Hagerman, Idaho, for consistent attention to meticulous detail during diet preparation, feeding, and care of the fish throughout the long bioassay. Mrs. Carlie Southard and Mrs. Dana Eshleman prepared, sectioned, and stained the material, and Mr. Charlie E. Smith helped review the many slides necessary to describe acute and chronic aflatoxicosis in the salmon and trout.

MATERIALS AND METHODS

Acute toxicity.—Groups of ten 25 g coho salmon fingerlings were force-fed gelatin capsules containing crystalline aflatoxin B_1 adsorbed on α-cellulose flour. These fish had been reared on a reference-complete test diet for fish nutrition studies (7) under constant temperature conditions (15°C) at the Hagerman Field Station of the Western Fish Nutrition Laboratory, Hagerman, Idaho, for approximately 6 months before treatment. Aflatoxin B_1 was dissolved in acetone, spread over a weighed amount of α-cellulose flour, mixed throughly into a homogeneous paste, solvent-evaporated overnight in darkness under reduced pressure, and batch-checked visually for uniform distribution of aflatoxin by short exposure under ultraviolet light. Calculated amounts of material were then weighed into small gelatin capsules which were stored in the cold in the dark until force-fed to individual fish.

Toxin dosage was programmed at 1, 3, 5, 10, or 15 mg/kg body weight for single dose, or at 1 and 3 mg/kg body weight/day for each of 5 days, or at 1 mg/kg body weight/day for each of 10 days of treatment, respectively. Negative controls were forced-fed once daily for 10 consecutive days with capsules containing only α-cellulose flour similarly treated with solvent, then dried before capsule loading.

Deaths were recorded as they occurred in each lot. Twenty days after the initial treatment, surviving fish were necropsied and any suspect tissue was preserved for subsequent histopathological review. Moribund fish were sampled, each was necropsied, details of internal organ anomalies were recorded, and then all suspect tissues were preserved for subsequent histology. Tissues selected for histological study were fixed in Bouin's solution, paraffin-embedded, sectioned at 6 μ, and stained with hematoxylin and eosin. A total of 109 salmon livers were histologically sampled for the acute toxic response to aflatoxin B_1. Fish were anesthetized for preliminary experiments by immersion in a solution of 1:25,000 tricaine methane sulfonate (MS 222, Sandoz), and then were intubated by forcing the gelatin capsule down the esophagus past the sphincter into the stomach with a glass rod. With proper handling, practice disclosed no need for anesthesia, and final acute toxicity experiments were conducted by grasping each fish with rubber-gloved hand, allowing initial struggle to subside, and then inserting individual capsule into opened mouth and down esophagus into stomach with glass rod.

Chronic toxicity tests.—Duplicate lots of 100 small coho salmon fingerlings, average weight 0.9 g each, were fed a complete test diet (CTD), previously demonstrated adequate to rear coho salmon or rainbow trout for at least 20 months in 15°C water without the appearance of any discrete nutritional deficiency syndrome or physiological anomaly (table 1). Negative-control fish were fed only this diet for 20 months, and test fish were fed toxin diet treatments of 1, 5, or 20 μg of crystalline aflatoxin B_1/kg dry diet ingredients for the same periods. Comparative tests were conducted with duplicate lots of 100 small rainbow trout fingerlings, average weight 0.6 g each, fed toxic test treatments at 20 μg of crystalline aflatoxin B_1/kg dry diet ingredient. Fish were fed 3 times daily, 6 days weekly at about 90% *ad libitum.* After fish grew to 3 g, diet was fed twice daily, 6 days weekly, for 6 months; thereafter, each diet was fed once a day for the remainder of the experiment.

TABLE 1.—Reference complete test diet*

Vitamin-free casein		38 parts
Gelatin		12
Corn oil		7
Cod liver oil		2
White dextrin		28
α-Cellulose mixture		9
α-Cellulose	8	
Vitamins†	1	
	9	
Mineral mixture		4
USP XII No. 2	100 g	
$AlCl_3$	15 mg	
$ZnSO_4$	300	
CuCl	10	
$MnSO_4$	80	
KI	15	
$CoCl_2$	100	
	100. 5 g	
Water		200
Total diet as fed (33% solids)		300

*Diet preparation: Dissolve gelatin in cold water. Heat and stir on water bath to 80°C. Remove from heat. Add while stirring: dextrin, casein, minerals, oils, and vitamins as temperature decreases. Mix well to 40°C. Pour into containers; refrigerate to harden; remove from trays and store in sealed containers in refrigerator until used. Consistency of diet adjusted by amount of water in final mix and length and strength of beating.

†Vitamin mix contains (in mg): thiamin · HCl, 5; riboflavin, 20; pyridoxine · HCl, 5; choline chloride, 500; nicotinic acid, 75; calcium pantothenate, 50; inositol, 200; biotin, 0.5; folic acid, 1.5; ascorbic acid, 100; vitamin B_{12}, 0.01; menadione (K), 4; and α-tocopherol acetate (E), 40.

Random samples of fish from each lot were selected periodically and fixed in Bouin's fixative for histological study. Internal organs of each fish sampled during the experiment were carefully examined for anomalies and each was recorded for subsequent correlation with histological review of that sample. Thirty fish were removed from each group on each diet treatment at 6 months on test, and laparotomies were performed on all survivors in every lot after 12 months on test. Technique for laparotomy resembled that performed on rainbow trout during hepatoma induction experiments reported elsewhere (*8*). Each fish was anesthetized, an incision was made at the midline between the pelvic girdle and the pectoral girdle, the incision was retracted, and viscera were carefully manipulated with probes and inspected for tumors or other anomalies. Each fish was tagged and number recorded with inspection results. After the wound was closed with gut suture, the fish was returned to recovery chamber until revived and then returned to the 6-foot circular tank for continued treatment. After 3 days, wound repair was well advanced and fish were fed. All surviving fish were necropsied after 20 months on test. At this time, each fish was exposed to a lethal dose of MS 222 and internal organs were carefully inspected while vascularized before the fish died. Representative random samples were removed for histological studies. All tissues were fixed in Bouin's solution and were later paraffin-embedded, skip-sectioned at 6 or 10 μ, and stained with hematoxylin and eosin to outline hepatoma nodules or other internal organ anomalies. For comparative purposes, equal numbers of rainbow trout exposed to the same maximum level of aflatoxin B_1 dietary insult were also sampled at 6, 9, 12, and 20 months for description of gross internal organ anomalies and comparative histology.

RESULTS

Gross Acute Aflatoxicosis

Deaths that occurred during tests with 25 g coho salmon fingerlings are shown in table 2. Day of insult is indicated for single and multiple consecutive days of insult. Sham-treated controls were normal in all respects, and at necropsy on Day 21 normal-appearing liver and other internal organs were observed. Fish fed 10 mg of aflatoxin B_1/kg body weight in single dose were slightly lethargic on Day 2–5 but were normal in external appearance and activity thereafter, and on Day 18 little if any gross damage was observed at necropsy.

Those fed 15 mg of aflatoxin B_1/kg body weight in single dose were more lethargic, turned dark for 3–5 days thereafter, and were not normally active until 10 days post treatment. At necropsy on Day 18, these fish showed some acute liver damage with small areas of hemorrhage scattered occasionally in small patches near the central anterior and posterior surfaces of the liver. Other internal organs appeared unaffected, and only mild residual toxicity effects could be attributed to this massive single dietary insult with aflatoxin B_1.

In contrast, salmon fed 1 mg/kg body weight/day for 10 consecutive days displayed extensive gross liver necrosis, areas of massive hemorrhage throughout the liver and extending into visceral fat, and bloody ascites within the abdominal cavity.

Similar effects were noted in salmon receiving 3 mg of aflatoxin B_1/kg body weight/day for each of 5 days, for a total dose of 15 mg aflatoxin B_1/kg. Most fish in both lots turned dark and became lethargic after 3–5 days of consecutive insult, and deaths began to occur by Day 10 in that

Table 2.—Treatment day and death of fish from aflatoxin

Aflatoxin B_1	Day 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Coho salmon																					
None	F*	F	F	F	F	F	F	F	F	F											T†
1 mg/kg bw‡	F										T										
3 mg/kg bw	F										T										
5 mg/kg bw	F										T										
10 mg/kg bw	F										T										
15 mg/kg bw	F										T										
1 mg/kg bw/day (5 mg)	F	F	F	F	F						T										
3 mg/kg bw/day (15 mg)	F	F	F	F	F						T										
10 mg/kg bw	F																	T			
15 mg/kg bw	F																	T			
3 mg/kg bw/day (15 mg)	F	F	F	F	F										3	2	1	1			T
1 mg/kg bw/day (10 mg)	F	F	F	F	F	F	F	F	F	F											
										2	2	2					2	2			
Rainbow trout																					
None	F	F	F	F	F						T										
3 mg/kg bw	F			3	3	2	1	1													
0.5 mg/kg bw/day (2.5 mg)	F	F	F	F	F	2	2	2	1	2	1										
0.1 mg/kg bw/day (0.5 mg)	F	F	F	F	F																
					1	1			2	1	T										

*F = fed.
†T = terminate and preserve all survivors for histology.
‡bw = body weight.

group receiving daily insult with aflatoxin at 1 mg/kg body weight/day. Similarly, mortality began to occur 10 days after 10 mg aflatoxin B_1/kg body weight total dose had been ingested, and deaths continued until the end of the 21-day test period. Three fish survived this treatment but obviously had been severely affected by the aflatoxin, and subsequent histology after Day 21 disclosed severe residual damage in the liver and other tissues.

Preliminary experiments disclosed groups of 10 salmon fed 3 mg aflatoxin B_1/kg body weight/day for 5 days or 1 mg aflatoxin B_1/kg body weight/day for 5 days were all alive by Day 10, and necropsy at this time disclosed only slight liver and other internal organ damage. That group administered a total of 15 mg of aflatoxin B_1 over a 5-day period had gross liver damage. The group receiving 5 mg of aflatoxin B_1/kg body weight over a 5-day period showed very little toxic liver damage when sampled and reviewed microscopically on Day 10. The LD50 for aflatoxin B_1 in 25 g coho salmon fingerlings appeared to fall between 5 and 10 mg of material/kg body weight, dependent upon continuous dietary insult with the toxin for 5–10 days.

Histopathology of Acute Aflatoxicosis

Sham-treated control coho salmon had essentially normal histology of liver (fig. 1) and of all other organs and tissues. Fish force-fed single doses of either 1, 3, 5, 10, or 15 mg of aflatoxin B_1/kg body weight and killed on Day 11 or 18 had progressively increasing degrees of prenecrosis of the liver. In those salmon treated only once with 3 mg of toxin/kg body weight, early stages of prenecrosis appeared. These livers showed loss of polarization of hepatocyte nuclei, some nuclear pleomorphism, and extensive cytoplasmic vacuolation (fig. 2). Fish fed 5 mg of toxin/kg 1 time had similar livers, but hepatocyte nuclei showed some chromatin margination and small patches of parenchymal cells with increased basophilia suggestive of beginning hyperplasia (fig. 3). In fish fed 1 mg of toxin/kg 10 times, more obvious hyperplasia with increased basophilia occurred, nuclear pleomorphism was more pronounced, and cytoplasmic vacuoles were larger. In some hepatocyte nuclei, the nucleoli were enlarged. Also, a trace of hyperemia and slight periportal lymphocytic infiltration were noted (fig. 4). Fish fed 3 mg/kg of toxin 5 times (maximum dose) had extensive areas of liver necrosis, but it was not zonal in distribution and was less massive than that seen in rainbow trout fed smaller doses of toxin either by single or multiple intubation. Reticulated strands of basophilic, hyperplastic hepatocytes occurred at highest insult and mitotic figures were common. Pyknotic and fragmented nuclei were also common in surviving and in necrotic hepatocytes (fig. 5). Hyperemia occurred in some coho livers but was less extensive and less severe than in livers of trout fed similar doses of toxin (fig. 6).

In other tissues, from fish fed either 1 mg/kg 10 times or 3 mg/kg 5 times, mild-to-severe edema and hyperemia occurred, particularly in visceral fat (fig. 7). Large masses of partially clotted blood sometimes were present in the intestine (fig. 8), the gills often showed moderate edema in the lamellae (fig. 9), the brain had meningeal edema (fig. 10), and in some salmon focal cerebral edema in white matter and foci of degenerating cerebral neurones were seen. Pyloric ceca had extensive sloughing of the mucosal epithelium (fig. 11), and the head kidney showed hyperplasia of the adrenal cortical tissue (fig. 12), which is normally limited to from one to a few layers of cells surrounding the larger blood vessels and sinuses. The excretory kidney often had cytoplasmic debris and occasional basophilic staining casts in the lumens of convoluted tubules. Sometimes pyknotic nuclei were common in the tubular epithelium, and glomeruli appeared shrunken, leaving large Bowman's spaces.

No consistent pathology occurred in esophagus, stomach, heart, spleen, or gonads. The pathology in coho salmon noted above for various tissues compared favorably with that previously reported for rainbow trout fed much smaller doses (0.1 and 0.5 mg/kg body weight) of aflatoxin B_1 (*4*). The most common liver lesion of acute aflatoxicosis was massive general hemorrhagic necrosis in trout and variable, patchy necrosis with hyperemic areas in those coho salmon fed 1 mg B_1/kg body weight 10 times or 3 mg B_1/kg body weight 5 times. Slight-to-moderate bile ductule proliferation occurred in aflatoxic trout and salmon livers, in contrast to the extensive proliferation of bile ductules in aflatoxic ducklings reported by Newberne *et al.* (*9*).

Chronic Aflatoxicosis

No coho salmon fed 20 μg of aflatoxin B_1/kg dry diet ingredients for 20 months developed any gross or microscopic liver neoplasia, whereas approximately 96% of trout fed the same diets for the same test period developed histologically confirmed hepatic cell neoplasia (*6*). The salmon were small compared to trout fed for equal periods the same test diet, and no grossly apparent or histologically detected liver anomalies were encountered in samples selected at 6, 9, and 12 months, or in surviving fish examined at necropsy at 20 months, then preserved, skip-serial sectioned, and examined microscopically. No other gross anomalies were observed in internal organs, and viscera were normal in size, shape, color, and general physiological appearance when sampled.

DISCUSSION

Coho salmon fingerlings did develop symptoms of acute aflatoxicosis when the toxin insult was increased 10–30 times the LD50 level effective for acute toxicity in rainbow trout of comparable size. Gross observation of samples of fish selected during the high-load test showed characteristically less extensive and generally less specific toxic liver or other visceral organ damage than that observed in rainbow trout. More time was necessary for acute effects in the salmon to become evident, and repetitive insult with the toxin was needed before frank toxicity was observed. Trout reacted within 2 or 3 days after a single massive dose, whereas the salmon showed gross and histological evidence of rapid liver cell regeneration and general elimination of gross effects 10–14 days post dietary insult. Only after 5–10 days' consecutive daily exposure to the toxin did death begin to occur, as did frank gross and histologically confirmed severe liver damage. These fish also still showed areas of regenerative hyperplasia reminiscent of recovery from partial hepatectomy (*10*). A poorly defined dose-response curve developed and, when the toxin was first fed for 5–10 consecutive days, an LD50 of 5–10 mg of aflatoxin B_1/kg body weight could be estimated for 25 g coho salmon held in 15°C water.

Chronic toxicity of aflatoxin in coho salmon fingerlings was not detected even when fish were fed 20 μg of aflatoxin B_1/kg dry diet ingredients daily for 20 months. No tumors developed in the salmon, whereas rainbow trout fed identical diets in parallel experiments developed typical hepatoma often with massive multiple tumors displacing or invading surrounding tissues by 20 months on test.

Coho salmon showed gross and histological evidence of rapid liver cell regenerative hyperplasia after dietary insult and appeared in long-term bioassay to be much more refractory to aflatoxin B_1 than young rainbow trout held under these experimental conditions (15°C water and 50% protein CTD).

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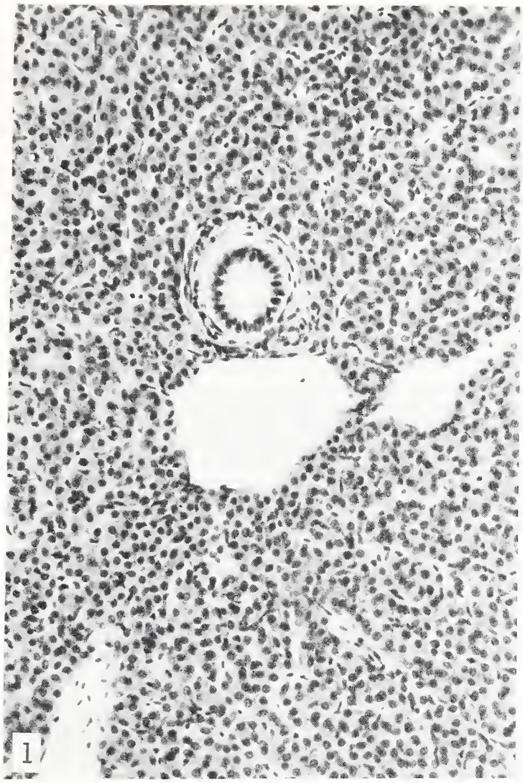
Figure 1.—Normal liver of coho salmon fingerling showing portal triad (*center*) surrounded by hepatic cords (muralia) averaging 2 cells in width and alternating with narrow sinusoids. Fed complete test diet. × 425

Figure 2.—Section of prenecrotic liver from salmon force-fed 3 mg/kg body weight of aflatoxin B_1 one time and killed on Day 11. *Note* partial loss of nuclear polarization, slight pleomorphism, and increased cytoplasmic vacuolation. × 265

Figure 3.—Prenecrotic liver from fish fed 5 mg/kg of toxin one time and killed on Day 11. Similar to figure 2, but with somewhat more nuclear pleomorphism, greater loss of nuclear polarization, increased cytoplasmic vacuolation, and a few small clusters of hepatocytes with increased basophilia suggestive of early hyperplasia. × 425

Figure 4.—Liver of coho salmon fingerling fed 1 mg/kg of toxin 10 times and sampled on Day 10, showing more advanced nuclear pleomorphism, pyknosis, enlarged nucleoli, enlarged cytoplasmic vacuoles, and a large increase in basophilic, hyperplastic cells, some including mitotic figures. × 425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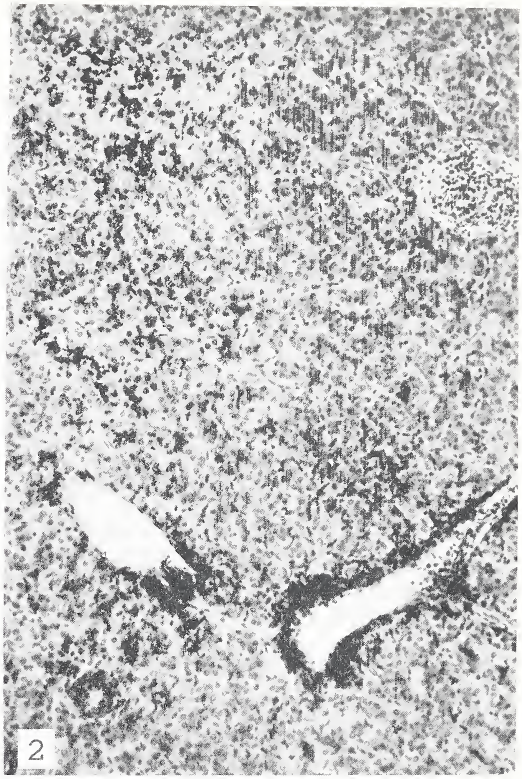
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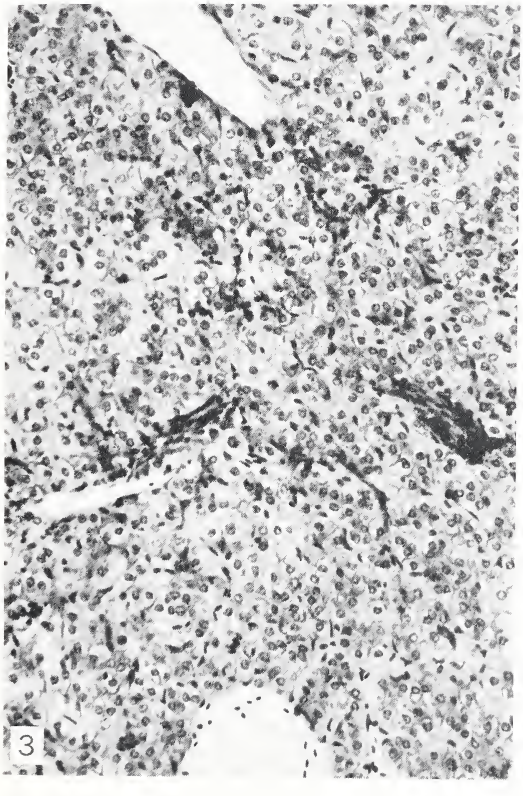
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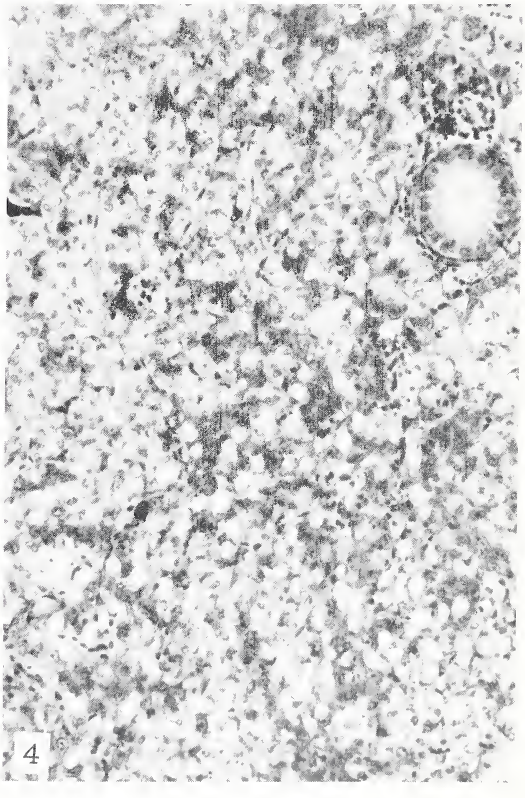
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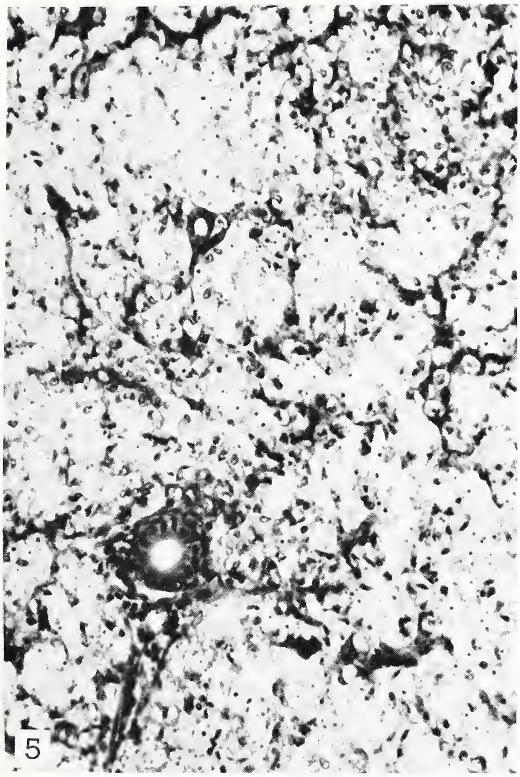
Figure 5.—Liver of fish fed 3 mg/kg of toxin 5 times (largest dose fed) and sampled on Day 15, showing patches of highly necrotic hepatocytes more or less surrounded by a reticular arrangement of hyperplastic, basophilic parenchyma cells, some of which included mitotic figures. × 425

Figure 6.—Hyperemia in liver of fish fed 3 mg/kg of toxin 5 times and sampled on Day 17. Surrounding the largest mass of nucleated red blood cells are hepatocyte nuclei showing chromatin margination and others near the periphery with pyknotic nuclei. A few groups of hyperplastic parenchyma cells are present, and some cells contain large cytoplasmic vacuoles. × 425

Figure 7.—Visceral fat adjacent to stomach wall, showing edema and hyperemia of the adipose tissue characteristic of acute aflatoxicosis in both rainbow trout and coho salmon. Fish fed 1 mg/kg of toxin 10 times and sampled on Day 17. × 165

Figure 8.—Small intestine of salmon fed 3 mg/kg of toxin 5 times and sampled on Day 17, showing mass of partially clotted blood in intestinal lumen. This condition was observed in numerous salmon and trout given high loads of aflatoxin B_1. × 165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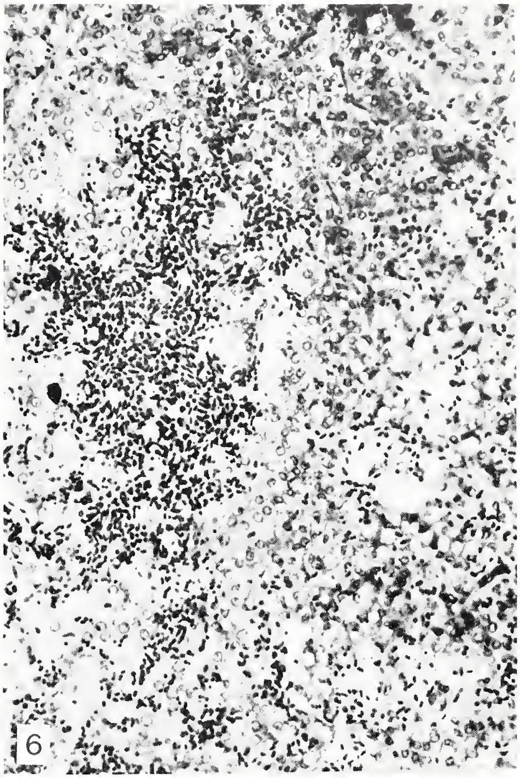
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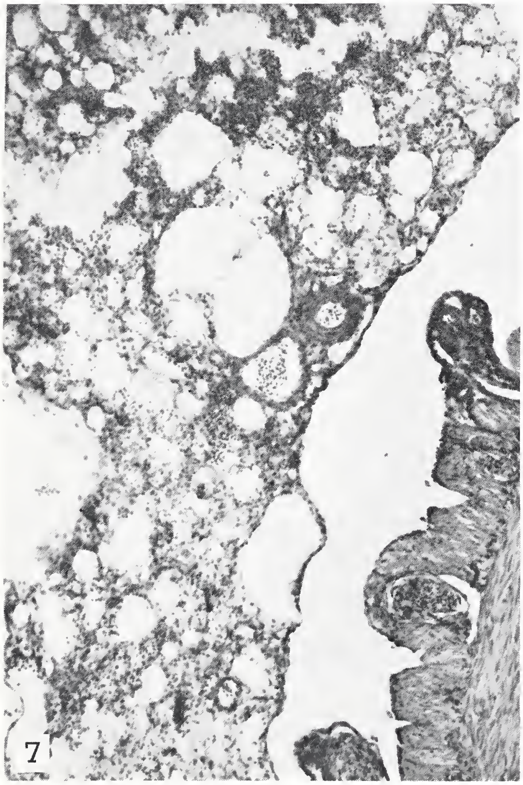
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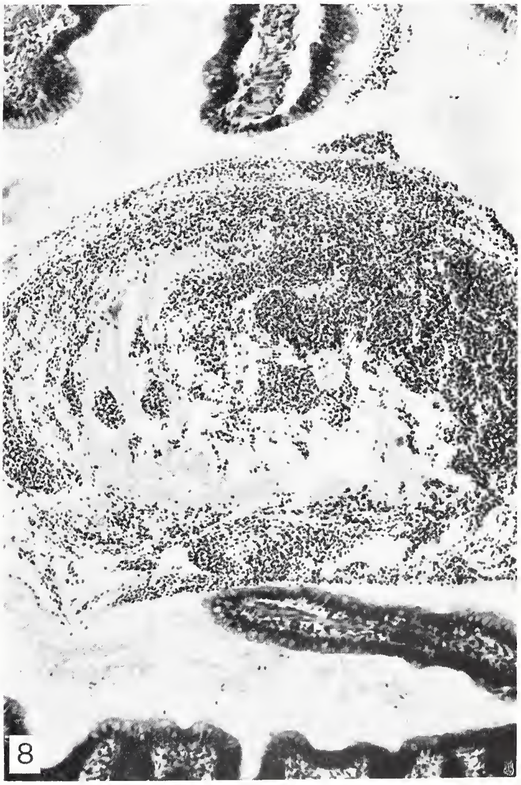
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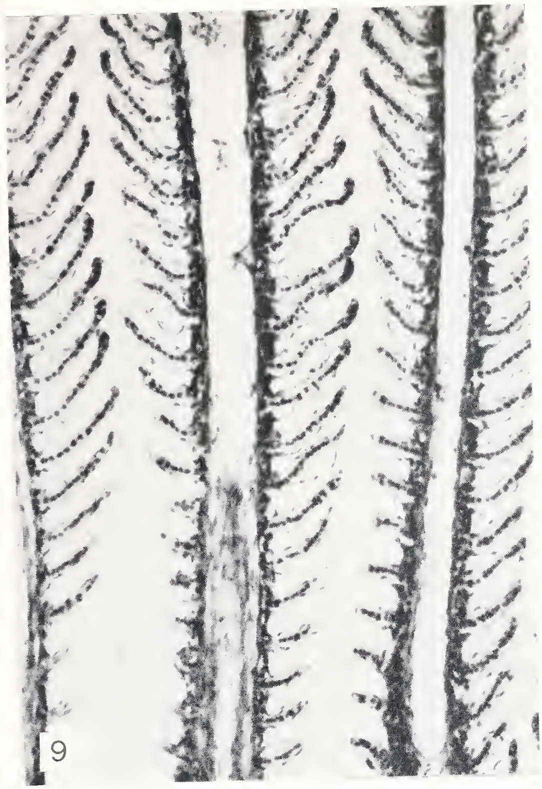
Figure 9.—Fish fed 3 mg/kg of toxin 5 times and sampled on Day 17 showing parts of 3 gill filaments. The delicate gill lamellae show a moderate edema wherever the delicate lamellar epithelium is lifted away from the central lamellar capillary. × 265

Figure 10.—Fish fed 1 mg/kg of toxin 10 times and sampled on Day 10, showing meningeal edema at the surface of the cerebrum in which traces of edema also occurred. × 265

Figure 11.—Extensive sloughing of mucosal epithelium in pyloric ceca and moderate hyperemia of "pancreatic" fat in salmon fingerling fed 3 mg/kg of toxin 5 times and sampled on Day 17. × 165

Figure 12.—Adrenal cortical hyperplasia in salmon fed 1 mg/kg of toxin 10 times and sampled on Day 17. Endocrine tissue is represented by the diffusely arranged pale cells—the darker cells are hematopoietic in character and contain among them a few melanin plaques. × 425



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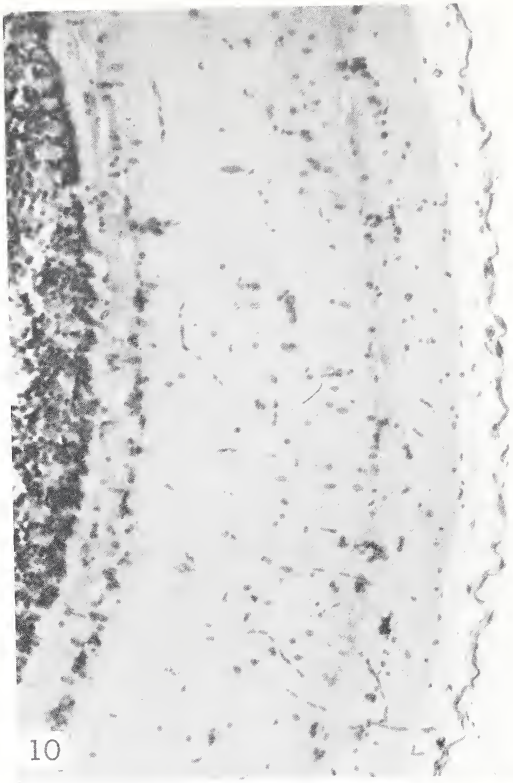

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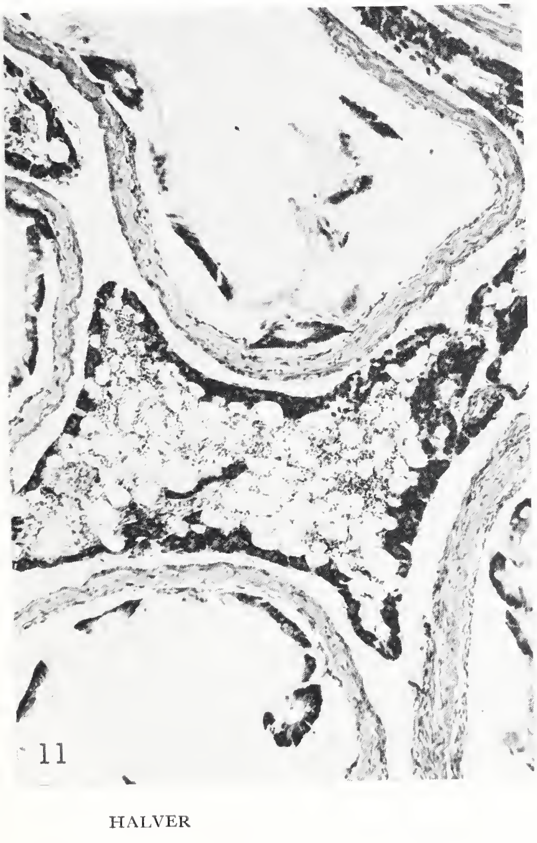

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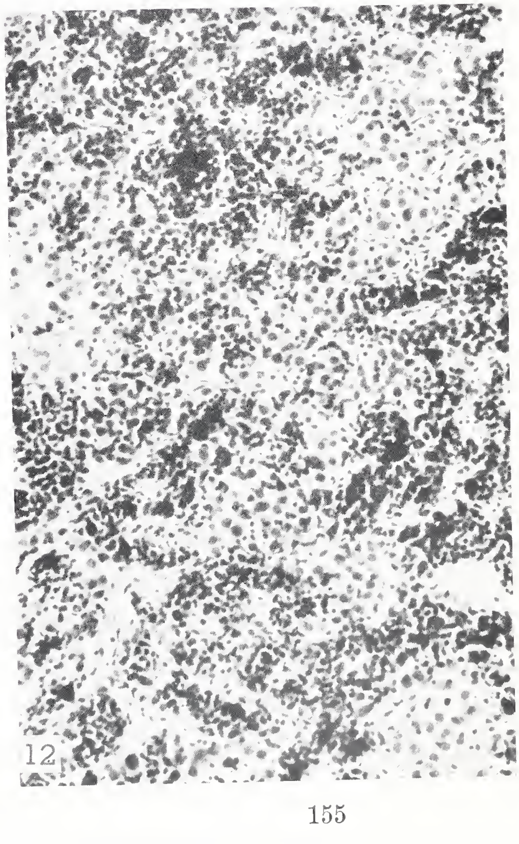

12

Case Reports of Three Teleost Neoplasms

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SUMMARY

A 6 × 4 × 2 cm reticulosarcoma loosely adhered to the stomach of a silver salmon *(Oncorhynchus kisutch)* which was about 25 inches long. Histologic sections, stained with either hematoxylin and eosin or Masson's trichrome or impregnated with Laidlaw's silver, support the diagnosis. A smooth, 5 × 4 × 3.5 cm, lobulated tumor was attached to a pectoral fin of a female coarse-scale sucker *(Catostomus macrocheilus)* 24 inches long. It was fibrous and quite vascular with angiomatous characteristics. Some blood spaces were cavernous. Although a diagnosis of fibroangioma seemed most fitting, the tumor also resembled a cavernous hemangioma in certain areas. A firm, ovoid, slightly lobulated neoplasm, measuring 15 × 9 × 7 mm, was found just caudal to the papillated margin of the upper lip of a male coarse-scale sucker 17.5 inches long. Histologic sections stained with hematoxylin and eosin revealed a fibrosarcoma with many atypical mitotic figures.—Nat Cancer Inst Monogr 31: 157–165, 1969.

CASE I

A PRIMARY solid tumor, 6 × 4 × 2 cm, was found loosely attached to the anterior peritoneal surface of the stomach in a mature ocean-caught silver salmon (*Oncorhynchus kisutch*) about 25 inches long and was diagnosed as a reticulosarcoma. It was promptly preserved in Bouin's fixative. Grossly, the tumor was firm, very thinly encapsulated, and smooth-surfaced except for variable-sized, shallow lobulations. The cut surface was nearly homogeneous in texture. Unfortunately no photograph was obtained of the gross specimen.

Histologic sections were cut at 6 μ and stained with either hematoxylin and eosin or Masson's or impregnated with Laidlaw's sliver. Microscopically, the tumor was poorly vascularized and of moderately variable density. The rather cellular stroma was a mixture of reticulum and fine collagenous fibers (fig. 1). The silver-impregnated sections showed numerous coarse and fine argyrophilic fibers, with the finer ones closely applied to reticulum cells (fig. 2). Masson's trichrome clearly differentiated the reticulum cells (purplish) and their fibers from the collagen fibers (green) and the fibroblast nuclei; it also showed mucin (pale green) deposits in scattered cells that were occasionally seen in groups resembling foci of fatty infiltration. Relatively typical mitotic figures were occasionally seen.

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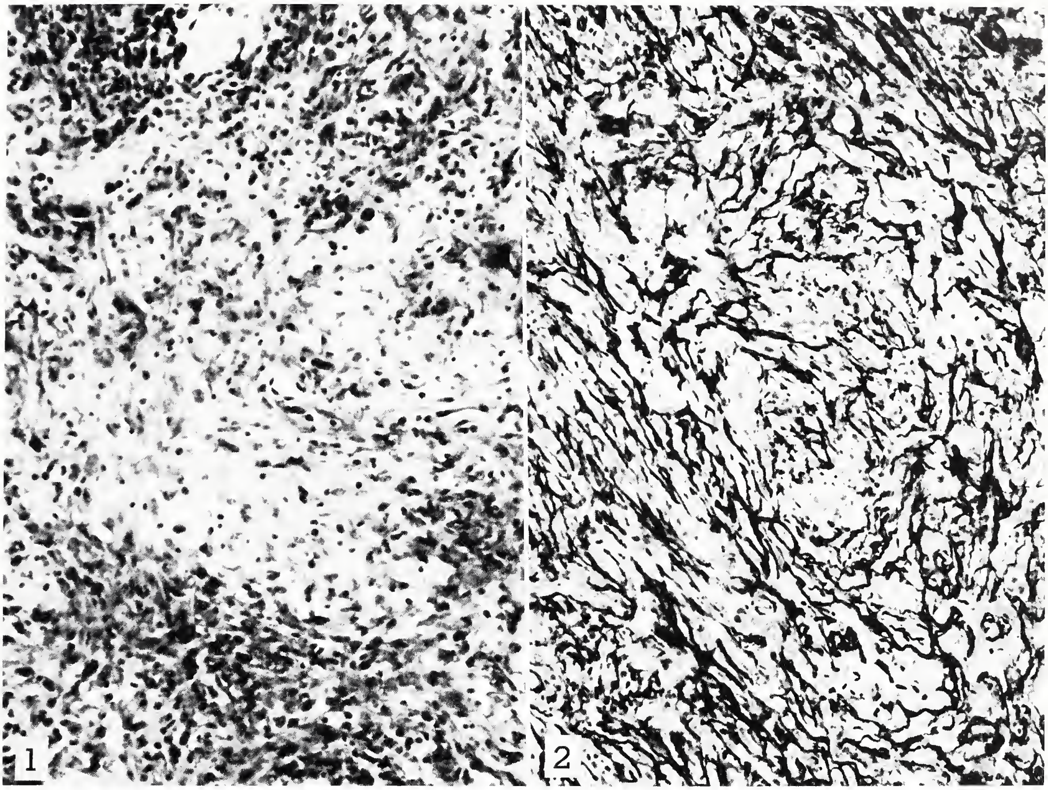
Tumors of any kind in salmon are rare, judging from the paucity of literature and from hundreds of silver salmon fingerlings fed 1, 5, or 20 ppb of aflatoxin B_1 for up to 20 months at this laboratory (WFNL). No tumors were found in these fish, some 50 of which have now been transferred for further observation from fresh water to sea water at the Bowman Bay Marine Station near Anacortes, Washington. After 12–20 months on test, rainbow trout fed 0.5, 1.0, and 20 ppb of aflatoxin B_1 had high incidences of liver cell carconima—the only neoplasms found in these fish. Of the 200–300 fish in each diet group, 50–100% developed hepatomas. The control trout remained essentially negative for hepatoma.

CASE II

A large, lobulated, but otherwise smooth-surfaced tumor, 5 × 4 × 3.5 cm, was attached to the anterior margin of the left pectoral fin of a female coarse-scale sucker (*Catostomus macrocheilus*) 24 inches long (fig. 3). This fish was taken from the Clark Fork River, 7 miles west of Missoula, Montana, on April 6, 1967, by L. G. Mitchell of the University of Montana and was reported in good condition except for the presence of the tumor. The neoplasm and attached fin were well preserved in 10% buffered neutral formalin when received at this laboratory (WFNL). Therefore, we did not know the consistency of the fresh tumor. Paraffin sections were cut at 6 μ and stained with hematoxylin and eosin, Masson's trichrome, Giemsa, mucicarmine, or periodic acid-Schiff, or impregnated with Laidlaw's silver. After histologic study, a diagnosis of fibroangioma was made. Histologically, the tumor was benign, overlaid with a scaleless, stratified squamous epithelium continuous with that of the fin surface. Just beneath the epithelium, the neoplasm was moderately cellular and finely fibrillar (fig. 4). This tissue became more coarsely fibrous and more or less angiomatous deeper from the surface. In some areas large, irregular, cavernous sinuses up to 4 mm in diameter were seen, which contained blood and/or coagulated blood plasma (fig. 5). The basic fibroangiomatous pattern varied considerably, some areas being relatively avascular. The denser stroma contained many collagenous fibers, whereas the less dense stroma resembled reticular connective tissue with more or less stellate cells and a very fine fibrillar stroma. Spindle-shaped fibroblasts were also common throughout. Mitotic figures were uncommon, and those seen were usually typical (fig. 6). Since this fibrous tumor appeared so obviously angiomatous, we diagnosed it as a fibroangioma, though certain parts of the tumor strongly resembled a sclerosing cavernous hemangioma.

CASE III

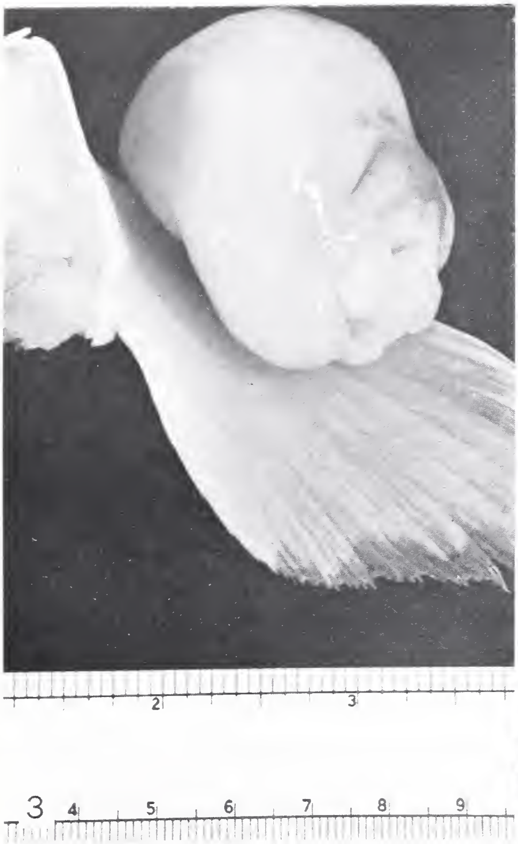
A small, slightly lobulated, ovoid smooth-surfaced tumor was found just behind the papillated margin of the upper lip of a male coarse-scale sucker 17.5 inches long (fig. 7). This fish, also collected by L. G. Mitchell, was caught in the Clearwater River at Clearwater Junction, Missoula County, Montana, on February 27, 1967. The neoplasm and a portion of the attached lip were preserved in 10% buffered neutral formalin before shipment to this laboratory (WFNL). After fixation, the tumor measured 15 × 9 × 7 mm, and upon sectioning, it was seen to be well lobulated throughout, firm, and finely fibrous. Histologic examination of paraffin sections, cut at 6 μ and stained with hematoxylin and eosin, Giemsa, or Masson's trichrome, revealed a fibrosarcoma with rather compact fibrous stroma and with many small, atypical mitotic figures, the chromosomes of which often appeared to contain swollen or vesiculated ends (figs. 8 and 9). Bizarre cells were common, but few giant cells were noted. The neoplastic stroma consisted mainly of poorly differentiated, more or less anaplastic fibroblasts with frequent spaces between them in some areas but with more compact fiber arrangements in others. Degenerating cells and cellular debris were occasionally present. Nuclear pleomorphism was widespread and conspicuous. Hyperchromatism occurred in parts of the neoplasm, but was subdued or absent in others. This appears to be the first reported case of fibrosarcoma in a coarse-scale sucker.

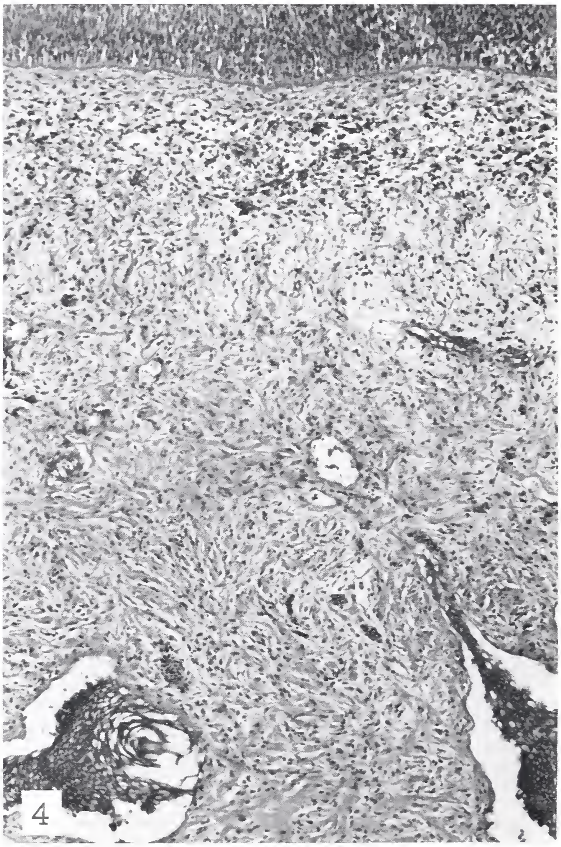


Case I (Figs. 1 and 2): Reticulosarcoma in Silver Salmon (*Oncorhynchus kisutch*)

FIGURE 1.—Reticulosarcoma from stomach wall of adult ocean-caught silver salmon. Stroma of reticulum and collagenous fibrils appears in *center* and a more collagenous stroma is present in the darker, peripheral areas. The highly cellular areas contain numerous lymphocytes and various other free cells. Hematoxylin and eosin. × 450

FIGURE 2.—Same as figure 1 but impregnated with Laidlaw's silver, showing coarse and fine reticulum fibers, the finer of which surround some individual reticulum cells. × 450

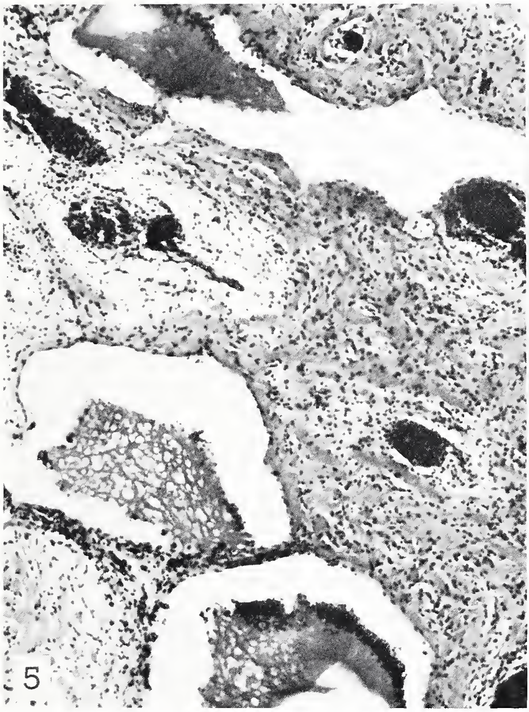




Case II (Figs. 3–6): Fibroangioma on Pectoral Fin of Coarse-Scaled Sucker (*Catostomus macroheilus*)

FIGURE 3.—Fibroangioma (5 × 4 × 3.5 cm) attached near anterior margin of the left pectoral fin. Photographed after formalin fixation. × 1

FIGURE 4.—Histologic section of fibroangioma showing epidermal stratified epithelium *above* a distinct basement membrane, *below* which the tumor occupies most of the dermal area. Small, blood-filled sinuses and cavernous sinuses containing coagulated blood plasma are common throughout most of the rather dense fibrous stroma. Hematoxylin and eosin. × 165



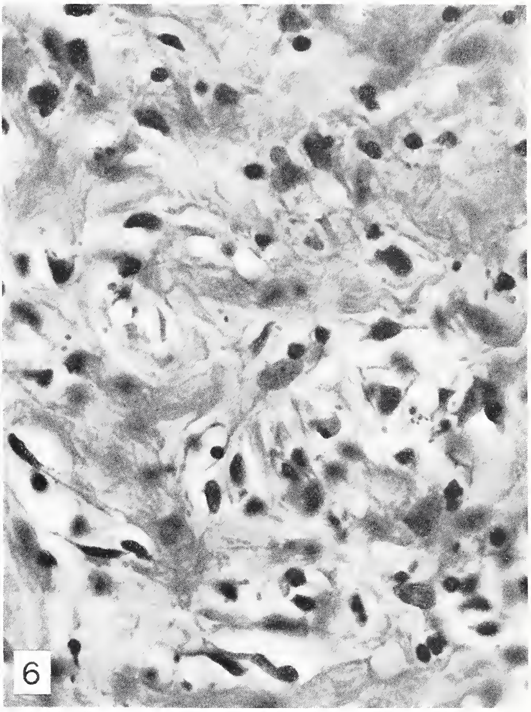
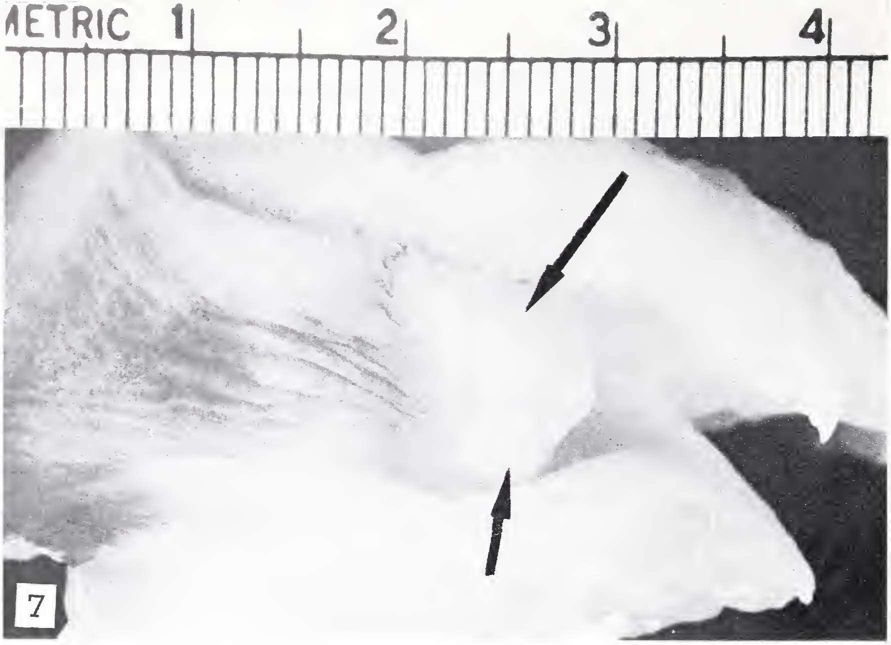


Figure 5.—Histologic section nearer center of tumor showing its cavernous angiomatous character. Hematoxylin and eosin. × 165

Figure 6.—High-power, showing somewhat anaplastic fibroblasts and numerous capillaries and small sinuses. *Black dots* may represent either pyknotic nuclear fragments or occasional erythrocyte nuclei. Hematoxylin and eosin. × 1000



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Case III (Figs. 7–9): Fibrosarcoma on Lip of Coarse-Scale Sucker

FIGURE 7.—Lip of coarse-scale sucker with fibrosarcoma (*arrows*). Tumor measured 15 × 9 × 7 mm after formalin fixation. × 3

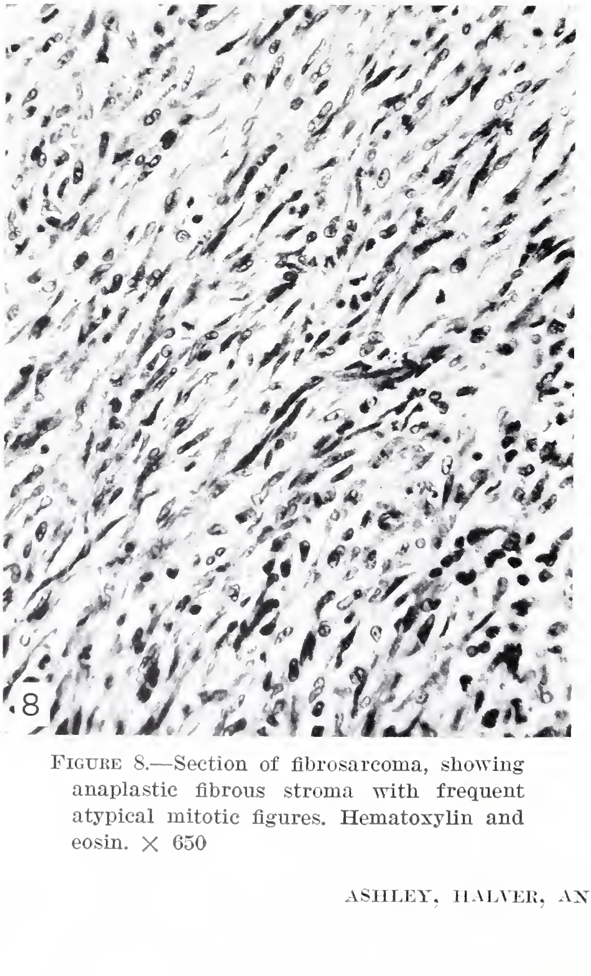

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FIGURE 8.—Section of fibrosarcoma, showing anaplastic fibrous stroma with frequent atypical mitotic figures. Hematoxylin and eosin. × 650

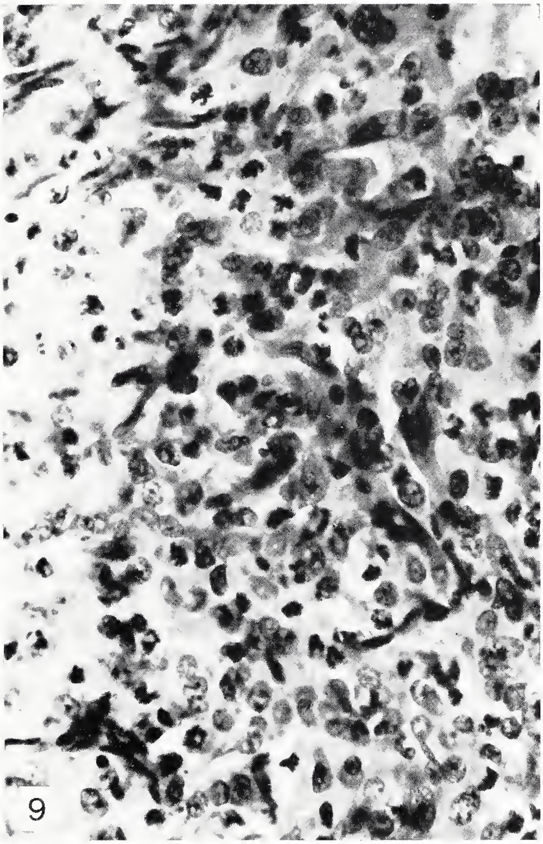

FIGURE 9.—Highly cellular portion of fibrosarcoma, showing numerous atypical mitotic figures, anaplastic nuclei, and giant tumor cells. Hematoxylin and eosin. × 2800

Lymphosarcoma of Possible Thymic Origin in Salmonid Fishes

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SUMMARY

Five cases of lymphoma in salmonid fishes are reported: three in brook trout, *Salvelinus fontinalis,* and two in the hybrid "splake" (brook trout × lake trout, *Salvelinus namaycush*). Two of the neoplasms in brook trout apparently arose in the thymus, and the third involved the thymus as well as the kidney. Whether the thymus was primarily involved in the splake is not known. All the cases were accompanied by leukemia, as estimated from histologic sections. The cell type in all was an immature lymphoid cell.—Nat Cancer Inst Monogr 31: 167–171, 1969.

A DISCUSSION of lymphoid neoplasms in fishes must be conducted with the understanding that circumscribed collections of lymphoid tissue in the form of nodes do not exist in these animals. In elasmobranchs, hematopoiesis takes place in the spleen, mesonephros, the submucosa of the intestine, and the subcapsular tissue of the gonad. In teleosts, it occurs primarily in the kidney, with the spleen and submucosa of the intestine having only a minor role. The kidney is active in hematopoiesis, and lymphosarcoma in this organ is found quite frequently in fishes. However, there is some evidence that the tumors discussed in this report originated in the thymus. In two of the five cases reported, the largest focus of tumor, apparently representing the primary tumor, was located in the area of the thymus, and other tissues and organs were secondarily involved either by direct invasion or through blood-borne metastases.

Schlumberger and Lucké (*1*) reviewed the tumors in fishes and listed 20 cases of lymphosarcoma in 9 species of fish. Only one case has been reported in salmonid fishes [Haddow and Blake (*2*)]. Nigrelli (*3*) reported 12 cases of lymphosarcoma in northern pike (*Esox lucius*), which seem to be highly susceptible to this tumor. Mulcahy (*4*) recently reported 13 cases of lymphosarcoma in this same species from Ireland. While several cases of lymphosarcoma were reported in other species of fish, it seems remarkable that only a single example has been reported in salmonids, since various species of this family are reared in large numbers by sport and commercial fisheries.

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[1] Mr. Dunbar died on March 23, 1969, following a myocardial infarction. He will be remembered by many friends and coworkers for his warm human qualities as well as for his enthusiasm in investigating the pathologic anatomy of diseases of fishes.

During the past several years, 3 yearling brook trout were found bearing small tumors ranging in size from 2–6 cm, and generally lying under the operculum (fig. 1). The tumors were snowy white and relatively soft. Because of the position of the nodules and the close resemblances between the tumor cells and thymic lymphoid cells, 2 of the 3 tumors appeared to originate in the thymus. The third tumor (fig. 2) was lying in the anterior portion of the body cavity and had greatly restricted the action of the heart. The heart, about half the normal size, had only a small space in which to perform its function (fig. 2). Figure 3 shows the dissected head, revealing the tumor in the anterior portion of the kidney, which was extensively involved by tumor continuous with that in the thymus. It is difficult to determine whether this tumor originated in the kidney, or in the thymic enlargement under the operculum. These 3 brook trout were all from a common strain of trout, namely, the Pisgah Forest strain, which is propagated extensively in the National Fish Hatcheries throughout southeastern United States. Although the number of tumor-bearing fish is low, it may reflect a genetical susceptibility of this particular strain of fish to lymphoma.

The two cases of lymphoma in the splake were submitted from Canada and were described grossly as small nodular tumors under the operculum.[2] Only stained sections of these tumors were received, but examination of these slides left no doubt as to the nature and type of tumor involved.

The significance of finding this type of tumor in a cold-blooded animal is clear, since it resembles closely certain leukemias in man. Figure 4 shows many circulating tumor cells (lymphoblasts), among which only a very few erythrocytes can be seen. The leukocytosis in the circulating blood most probably represents vascular spread of the tumor cells, rather than a cellular reaction to the neoplasm because the cells in the blood vessels are morphologically identical with those in the tumors.

Macroscopically, tumors in the brook trout were not large. The tumor in figure 1 measured 2 × 2.5 cm. The dorsal one-third of the gill cavity was occupied by the tumor, forcing the gill arches down and out. Broad bands of tumor cells were invading the adjacent musculature, separating and destroying the muscle bundles (fig. 5). The tumor in figure 2 measured about 3 × 6 cm and had spread throughout the kidney, greatly enlarging this organ. The musculature around the head kidney was invaded in every direction. The liver was slightly swollen and contained small white perivascular nodules consisting of lymphoblasts (fig. 4).

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[2] Personal communication, Dr. Joan Budd, University of Guelph, Guelph, Ontario, Canada.

The tumor in figure 3 protruded from under the operculum and invaded and enlarged substantially the head kidney. As in the other tumors, the musculature, the liver, and blood were invaded by many tumor cells.

Microscopically the tumors were composed of large, round, uniform lymphoblasts, bound into trabeculae by very thin strands of fibrous tissue. Wide sinusoidal spaces separated the sheets or cords of tumor cells (fig. 6) and may have represented lymph channels [Johnstone (*5*)]. The chromatin of the tumor cell nuclei showed a tendency to become marginated, or condensed into solid masses. The nuclear membranes were very distinct. Mitotic figures were numerous in these apparently rapidly growing tumors. No bizarre cells were seen in any of the 3 cases. Cell uniformity was the most striking common characteristic in all the tumors.

Focal necrosis was often observed in areas of muscle invasion. The posterior kidneys were invaded by the tumors, and normal kidney epithelial cells were scarce. Focal necrosis of some individual collecting tubules was seen (fig. 7).

Metastatic nodules were extensive in the livers; most were microscopic in size, but the areas around practically all larger vessels were invaded quite extensively (fig. 6). The sinusoidal spaces throughout the livers contained tumor cells.

Microscopically, the lymphomas in the splake appeared identical to those in brook trout. The tumors spread primarily by invasion of the surrounding tissues. Figure 8 shows a few muscle fibers surrounded and being destroyed by the invading tumor. The individual tumor cells were slightly smaller than the tumor cells in the brook trout, but the general characteristics were the same. No details were submitted with these specimens regarding spread to the liver and other visceral organs, nor was there any information regarding the number of circulating tumor cells.

Because of the cytologic similarities between the tumors in brook trout and in splake, and because such tumors have not been reported in lake trout, it seems likely that a predisposition of splake to develop thymic lymphoma is genetically transmitted from the brook trout.

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(*1*) Schlumberger, H. G., and Lucké, B.: Tumors of fishes, amphibians and reptiles. Cancer Res 8: 657–753, 1948.

(*2*) Haddow, A., and Blake, I.: Neoplasms in fish: A report of six cases with a summary of the literature. J Path Bact 36: 41–47, 1933.

(*3*) Nigrelli, R. F.: Spontaneous neoplasms in fishes. III. Lymphosarcoma in *Astyanax* and *Esox*. Zoologica 32: 101–108, 1947.

(*4*) Mulcahy, M. F.: Lymphosarcoma in pike *Esox lucius* L (Pisces; Esocidae) in Ireland. Proc Roy Irish Acad (B) 63: 103–109, 1963.

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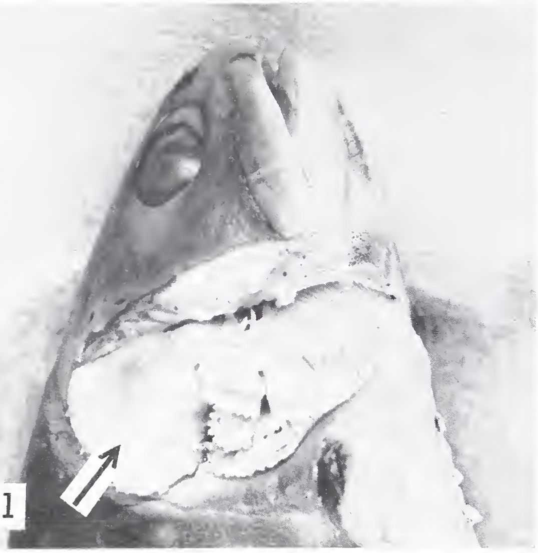
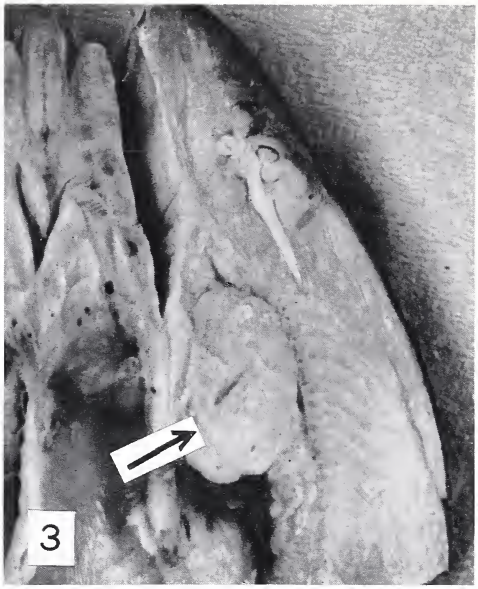
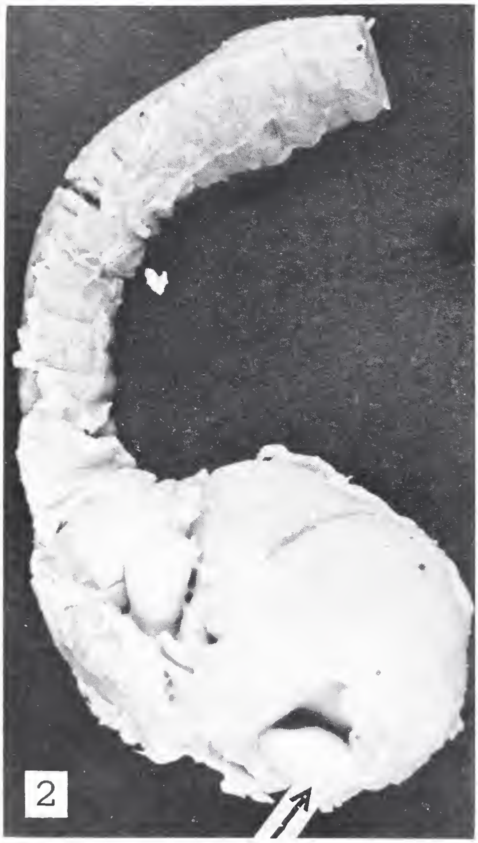
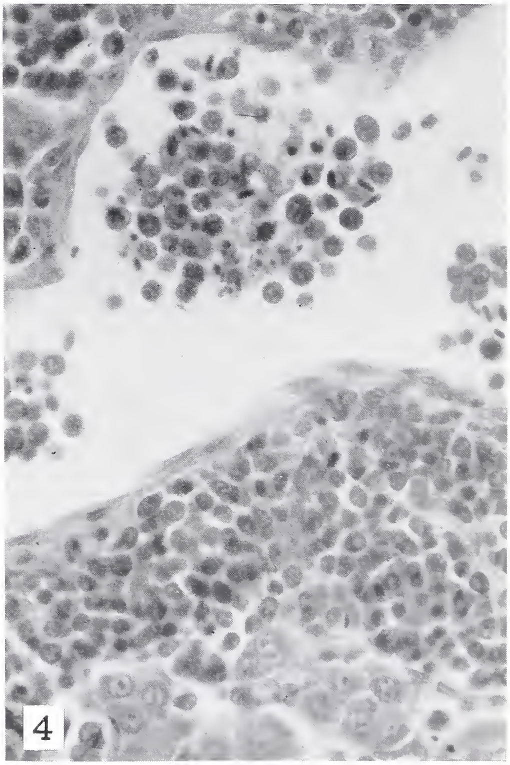








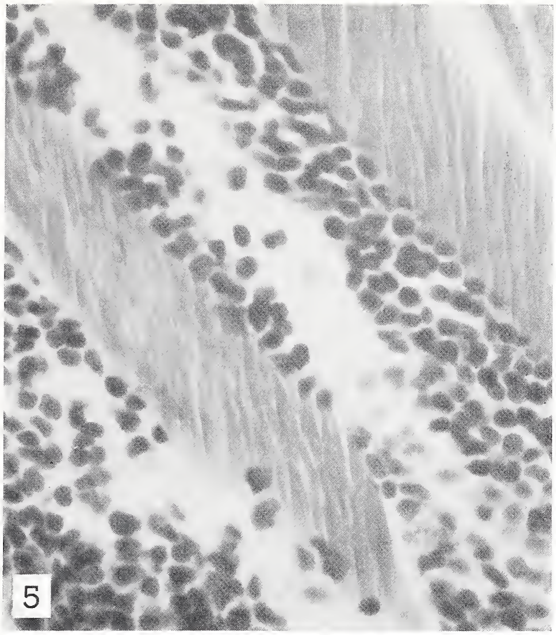
All sections were stained with Harris' hematoxylin and eosin.

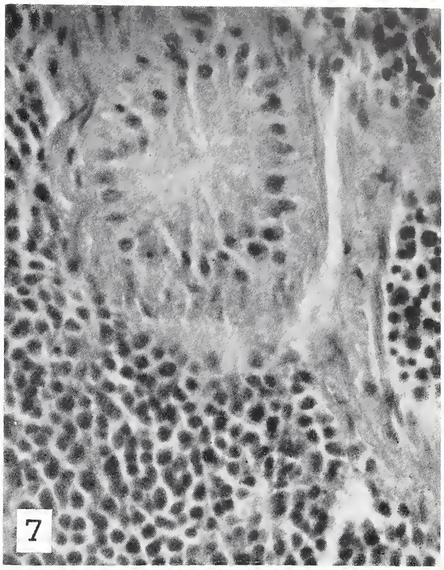
FIGURE 1.—Lymphosarcoma nodule occupying upper one-third of gill chamber (*arrow*) in yearling brook trout.

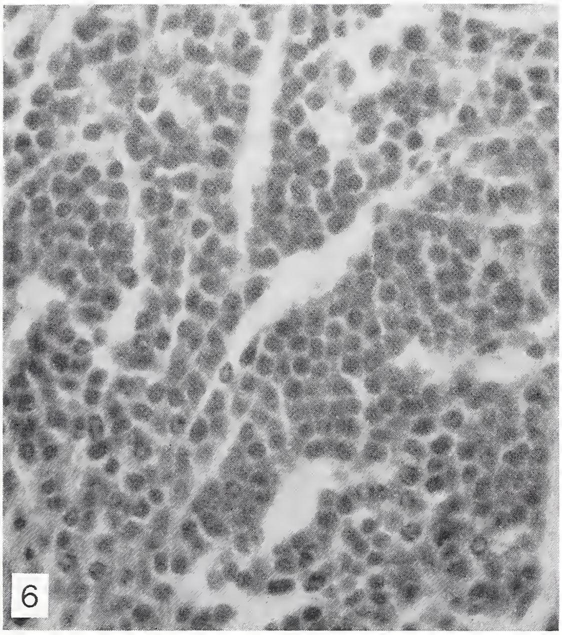
FIGURE 2.—Tumor nodule in anterior kidney of yearling brook trout. This tumor completely filled the cardiac chamber, and the pressure it applied to the heart had greatly restricted its size (*arrow*).

FIGURE 3.—Head of yearling brook trout dissected to show tumor enlarging the anterior kidney (*arrow*).

FIGURE 4.—Large central vein in liver of yearling brook trout. *Note* large number of circulating lymphoblasts. Perivascular spreading is adjacent to the vessel.







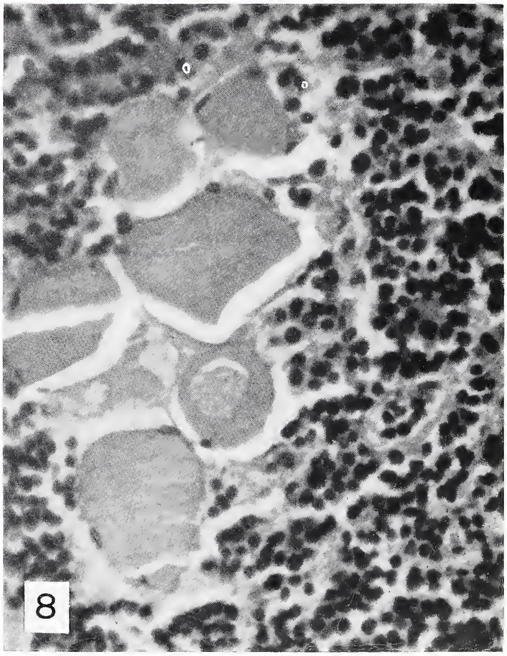


FIGURE 5.—Tumor cells invading and destroying muscle tissue around primary tumor nodule in thymus of yearling brook trout.

FIGURE 6.—Section of primary tumor in yearling brook trout, showing broad trabeculae of tumor cells separated by wide sinusoidal spaces.

FIGURE 7.—Section through posterior kidney of yearling brook trout, demonstrating necrosis of tubules and replacement of hematopoietic tissue by tumor cells.

FIGURE 8.—Section through outer edge of tumor in "splake" thymus, showing remains of a few muscle fibers.

Epizootiology of Papillomas in English Sole, *Parophrys vetulus*[1]

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SUMMARY AND CONCLUSIONS

This study concerns the epizootiology of papilloma-like tumors in the English sole, *Parophrys vetulus*, of San Francisco Bay. The neoplasms are best described as epidermal papillomas and appear similar to those found in a small percentage of several common flatfish inhabiting intertidal areas of the North Pacific Coast. An important difference, however, is the high incidence and specificity of the disease for English sole found in San Francisco Bay. This high incidence and a significant increase in percent of tumorous fish from South to North Bay indicate a relationship between certain environmental factors such as industrial waste discharge, salinity, and depth and susceptibility to the disease. Collected data on over 15,000 English sole have been analyzed and presented. In many respects, there is very little difference between normal and tumorous fish; however, there is strong evidence that the time of birth and the growth rate of tumorous fish are significantly different than for normal fish. This difference in growth rate cannot be directly associated with any debilitating effect of the disease. It is hypothesized that fish with tumors may belong to a stock different from that of the nontumorous fish. The data thus far collected indicate that two populations of English sole in San Francisco Bay are defined by the presence of tumors and growth characteristics. Whether these two populations are genetically distinct as subpopulations or are essentially the result of environmental factors remains to be determined.—Nat Cancer Inst Monogr 31: 173–185, 1969.

IN RECENT YEARS, various California State agencies interested in fish, game, and water quality have become concerned with tumorous epidermal lesions appearing on large numbers of the English sole, *Parophrys vetulus*, in San Francisco Bay. This concern was brought to our attention

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[1] This work was supported in part by funds from three sources: the University of California Cancer Committee, Project 1797; the Northern California Section of the American Cancer Society; and Public Health Service grant SO 1 FR-5441-08 from the Division of Research Facilities and Resources. We are also greatly indebted to the Marine Resources Operations Laboratory, California State Department of Fish and Game, and to Dr. D. Pappagianis, Professor of Medicine, University of California, Davis, Calif., whose help in describing these epidermal lesions was invaluable.

by the California State Department of Fish and Game in 1964, at which time we began to actively investigate the disease.

The English sole, also commonly known as "sole," "California sole," and "Lemon sole," is the only species of the genus *Parophrys*, family Pleuronectidae. These fish attain a maximum length of approximately 45.7 cm and have a lifespan of 15–20 years. They are distributed along the Pacific Coast of North America from Sitka, Alaska, to Santa Barbara, California, and perhaps as far south as Baja, California. English sole spawning begins in November, is heavy in December and January, diminishes in February, and is usually completed in March. Spawning preferentially occurs in shallow water. It is suspected that San Francisco Bay may be a spawning or nursery ground for immature English sole. It was speculated by Skinner (*1*) that the flatfish of the Bay may represent a discrete population exhibiting little exchange with ocean stock.

One of the earliest references to this disease in San Francisco Bay was made by Harold and Innes (*2*) who stated that as early as 1922 there was a great abundance of juvenile English sole among fishes encountered in San Francisco Bay; the occurrence of wartlike dermal swellings on a proportion of these fish was observed. These lesions were described as very similar to common warts, and it was suggested that the etiology might be viral.

Except for the work herein reported, the most active investigations concerning this disease in flatfish have been conducted in the Puget Sound area of British Columbia, by Wellings *et al.* (*3*, *4*) and by Nigrelli *et al.* (*5*). These workers, although primarily interested in flathead and sand sole, reported the occurrence of this neoplastic disease, in English sole. Wellings and Chuinard (*6*) suggested that the disease, in flathead sole, has a viral etiology, since electron photomicrographs of tumorous material revealed virus-like particles and associated granular bodies that are not observed in normal epidermis.

TUMOR DESCRIPTION

The most obvious type of tumor seen was cauliflower-like in elevation and appearance. Figure 1 is a composite photograph of four fish bearing these epidermal tumors. The tumors seem to progress from small hemorrhagic nodules a few mm in diameter to a larger, more diffuse, granular lesion, to a raised, often stalked, very wartlike tumor. They ranged from a few mm to as large as 50 mm in diameter. Histologically, they seem to fall into three related groups:

1) A papillary tumor consisting of bundles of epidermal cells with a central connective tissue column that is continuous with a connective tissue base lying parallel to the surface and appearing somewhat well organized but apparently not penetrating the connective tissue base.
2) A tumor that appears to consist of a connective tissue stroma in which many blood channels—venules or capillaries—lie. Its surface lacks a defined epidermal

covering, though a thin noncellular cuticular layer may be present. Again, obvious invasion of underlying structures was not seen.

3) A tumor, with a papilloma-like appearance, consisting of a disorganized structure resembling chronic inflammation or necrotic tissue.

Mitotic figures have not been conspicuous in any of the tissue sections studied. Both macroscopically and microscopically, these tumorous lesions seem similar to those described by Wellings *et al.* (*3*, *4*) and by Nigrelli *et al.* (*5*) in their studies of tumors occurring in various flatfish taken in North Pacific Coast waters.

EPIZOOTIOLOGICAL STUDIES

Sampling and Statistical Methods

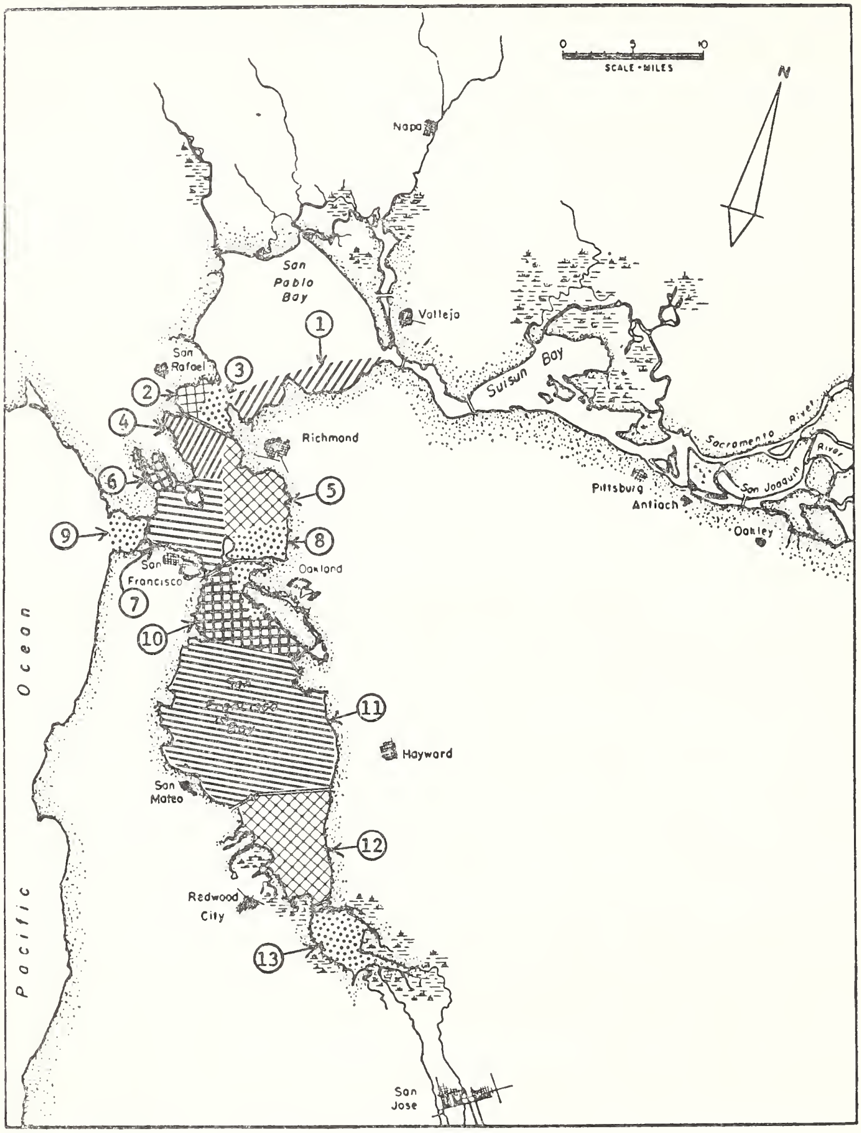
For approximately 1 week each month during the year, September 1965 to September 1966, fish were collected from San Francisco Bay with a small otter trawl. Sampling areas were defined by their basic topography and hydrography and in light of available pollution data. The areas are shown in text-figure 1.

Every English sole taken from San Francisco Bay during the sampling period was frozen and taken to the laboratory where the tumor-bearing fish were segregated from the nontumorous fish. Each fish was given an identification number, counted, weighed, and measured (standard length). In addition, the affected fish were carefully examined for the number of tumors present. Each tumor was described in terms of location on the fish and its elevation, pigmentation, texture, vascularity, and size.

All the data concerning English sole taken from San Francisco Bay were entered on IBM cards. A coding system was devised which would facilitate statistical analysis with both the IBM 1620 and 7090 computers. The information on each card included: card number, fishing area, date, time, sample area, tidal current, trawl time, total number of fish taken, fish identification number, standard length (mm), weight (g), number of tumors, tumor identification number, tumor location (body area, head, tail, fin), tumor elevation, vascularity, pigmentation, and size.

A standard cross-tabulation program was used to compile the large amount of data into a number of meaningful tables, which facilitated further analyses. Where applicable, regular regression analyses, Chi-square values, and Student's *t* tests were determined by use of standard computer programs.

The significance of the various parameters in relation to tumorous and nontumorous fish was determined by means of a stepwise, multiple regression analysis as outlined in the BMD-02R computer program (*7*). Later in the study, more sophisticated statistical analyses of fish growth characteristics were made with an asymptotic regression analysis proposed by Stevens (*8*).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Redwood City
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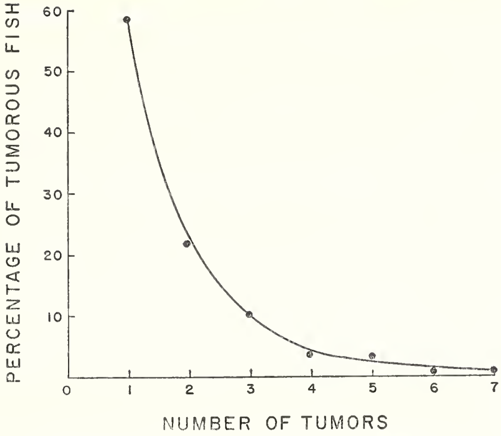
San Jose

TEXT-FIGURE 1.—Sampling areas in San Francisco Bay.

RESULTS

During this study, 15,739 English sole were taken from San Francisco Bay. Twelve percent of these were tumorous, with as many as 33 tumors per fish. Depending upon time of year and geographic locations, the proportion of tumorous fish ranged from 3–28%. The frequency distribution of number of tumors per tumorous fish is shown in text-figure 2. Over



Text-figure 2.—Frequency distribution of number of tumors per tumorous fish, San Francisco Bay.

58% of the tumorous fish were affected with 1 tumor, 22% with 2 tumors, 10% with 3 tumors, and an increasingly small proportion with 4 of more tumors.

The location of 2,194 tumors on the fish surface was analyzed. Thirty-one percent were located on the top body surface, 35% on the bottom body surface, 26% on both sides (always a fin), and 7.8% were classed as other, which includes lip, jaw, and incomplete data. The differences in these percentages are not significant.

The interrelationships of the gross appearance of the various tumors seen were examined using correlation analysis. Table 1 is the correlation matrix of these tumor description parameters. In general, these data show the highest degree of relationship between location of the tumor on the fishes' surface and pigmentation, between pigmentation and texture, and between elevation and texture. A correlation (not shown in table 1) was also seen between tumor size (area) and texture.

Table 1.—Correlation matrix for tumor description parameters

	Location	Elevation	Blood	Pigment	Texture
Location	100	28	22	53	24
Elevation	28	100	29	47	52
Blood	22	29	100	33	31
Pigment	53	47	33	100	57
Texture	24	52	31	57	100

The frequency distribution of normal and tumorous English sole in San Francisco Bay is shown in text-figure 3. In both instances, the average fish length is between 80 and 89 mm. The distributions are quite similar, except that the normal fish show less scatter around the mean length.

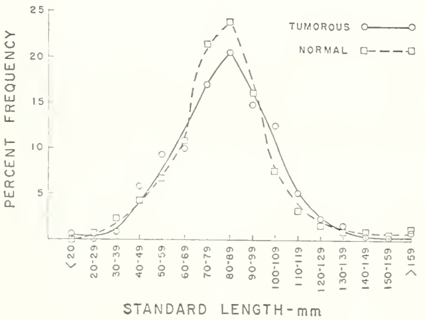
The weight-length relationship between normal and tumorous fish was determined to be identical and fit the equation:

$$W=kL^b \qquad [1]$$

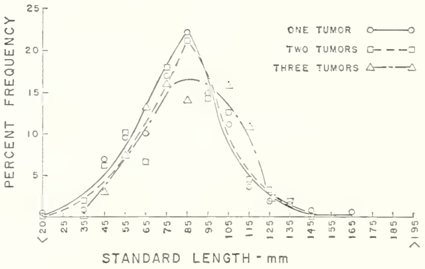
in which W is the fish weight, k and b are constants, and L is the fish length. Regardless of whether the fish is tumorous or not, the constants are the same.

The size frequency distribution of tumorous fish with 1, 2, and 3 or more tumors is shown in text-figure 4. The size distribution of fish with 1 and 2 tumors is practically identical, while the distribution for fish with more than 3 gives approximately the same mean length as the others but is skewed to the right.

When the median standard length of tumorous and nontumorous fish was examined for each month, in each trawl area, there was a marked tendency for the tumorous fish to be larger than the nontumorous fish during approximately the last three-fourths of the study year. Text-figure 5 represents the length frequency distribution of normal and tumorous fish by month. It is readily apparent that beginning in May the size of both tumorous and nontumorous fish steadily increases, with the tumorous fish ultimately exceeding the size of the nontumorous fish. The small proportion of larger fish in each month is probably from an earlier season. A plot of median fish length against month for the entire Bay generated

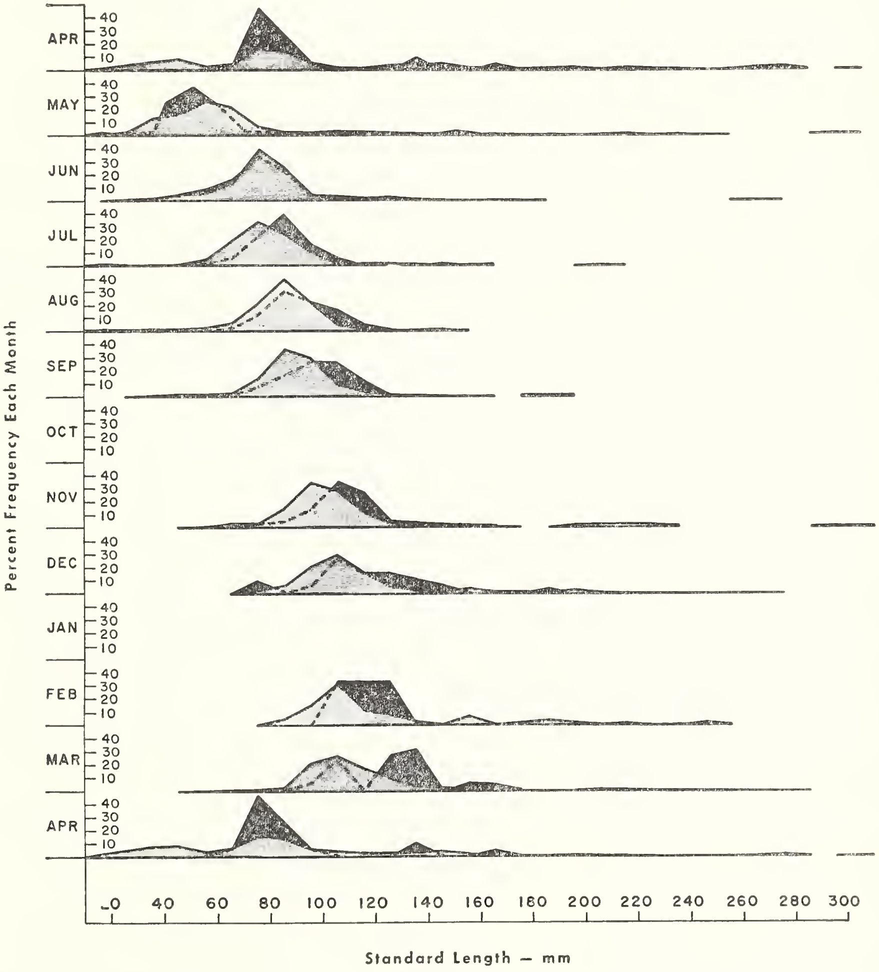


Text-figure 3.—Frequency distribution of normal and tumorous fish, San Francisco Bay.



Text-figure 4.—Frequency distribution of the standard length of English sole with 1, 2, and more than 3 tumors per fish.

two curves that indicate that growth was asymptotic and that tumorous fish grew differently than nontumorous. To test the significance of these two curves, an asymptotic regression analysis was performed. The accomplishment of this rather complicated reiterative analysis required that the bias of larger fish remaining from a previous season be removed. Size ranges were selected for each month on the basis of the length distribution (text-fig. 5) and are shown in table 2. A sample of tumorous and nontumorous fish was selected for analysis. Because of the large number of fish involved, the square root of the number of normal cases was chosen as a representative sample; and, because of smaller numbers, one-half of the tumorous cases was selected from within the proper size ranges.



TEXT-FIGURE 5.—Standard length frequency distribution of normal and tumorous fish by month. *Solid black areas* = tumorous fish; *shaded areas* = nontumorous fish.

Table 2.—Size range for asymptotic regression analysis

Month	Size range (mm)
April	10–55
May	20–85
June	25–105
July	35–105
Aug.	45–115
Sept.	65–120
Nov.	75–125
Dec.	80–135
Feb.	75–145
March	85–155

The results of the regression analysis are shown in table 3. The asymptotic growth curve is expressed as:

$$Y = A + BR^{x} \qquad [2]$$

in which Y is the fish length at time t; A is the asymptotic length; x is the time in months; B and R are functions of growth rate. Equation [2] is very similar to the well-known von Bertalanffy growth equation:

$$L_{t} = L_{\infty} - L_{\infty} e^{kt_{o}} \cdot e^{-kt} \qquad [3]$$

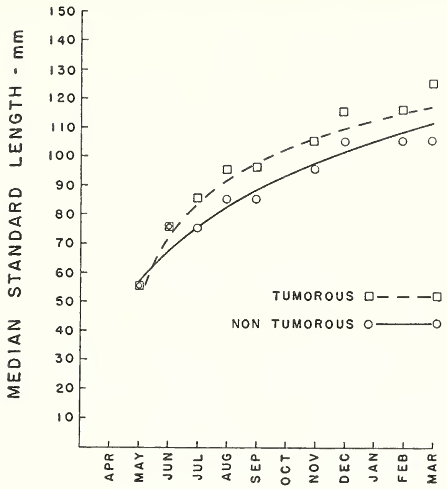
in which L is the fish length (Y in equation [2]); L_{∞} is the asymptotic (A); $L_{\infty}e^{kt_{o}}$ is the equivalent to B; and e^{-k} is equivalent to R.

The curves generated by these equations are shown in text-figure 6. The points indicated are actual median length values for all sole taken during the study. Median length was used because it is not sensitive to extreme values. The t values indicate that there is no significant difference between the asymptotes of the two curves but that the differences between the B and R values are significant at the 95% confidence level.

Ideally, each Bay trawl area should have been sampled at the same time to correct for the influence of seasonal variation on differences observed from one trawl area to the next. This was logistically impossible; therefore, a stepwise regression analysis was preformed to determine the most important factors affecting the proportion of tumorous fish in each sample. This analysis indicated that 30% of the variation in the propor-

Table 3.—Asymptotic regression analysis and "t" values for fish length vs. month

Fish	A	σA	B	σB	R	σR
Tumorous	119. 9	3. 1	−62. 6	2. 9	0. 72	0. 023
Nontumorous	123. 6	5. 6	−78. 6	4. 6	0. 82	0. 025
t test	0. 58		2. 96		2. 94	



TEXT-FIGURE 6.—Calculated asymptotic growth curves for tumorous and nontumorous fish. *Points shown* are actual median length values of all fish examined.

tion of affected fish in each sample could be accounted for by time of year and geographic location; only 12% could be accounted for by time of year alone. This would suggest that variation seen in the incidence of this disease was not unduly affected by the periodicity of sampling. Tide, current, depth, etc., had no effect on variation of data.

When the 13 trawl areas were compared to each other, the differences observed were difficult to assess. The various areas were then combined into North Bay (areas 1–5, text-fig. 1) and South Bay (areas 6–9). Very few

TABLE 4.—Total number of English sole taken each month in the North and South trawl areas of San Francisco Bay

Month	Number of fish taken			
	North area		South area	
	Total fish	Tumorous (%)	Total fish	Tumorous (%)
Feb.	66	4. 5	22	0
March	14	0	595	3. 0
April	118	9. 0	37	5. 4
May	2, 220	14. 1	237	4. 2
June	2, 251	15. 1	1, 332	10. 0
July	232	28. 4	2, 084	8. 0
Aug.	328	27. 1	1, 899	10. 0
Sept.	1, 442	16. 8	1, 495	13. 6
Nov.	457	10. 7	574	3. 8
Dec.	72	11. 1	179	5. 6
Total	7, 200	15. 5	8, 454	8. 9

fish were taken farther south than trawl area 9 and none north of area 1. The number of English sole taken from the north and south areas and the proportion of tumorous fish from each area are shown in table 4. As indicated, the incidence of tumorous fish in the north area is approximately twice that encountered in the south area. A *t* test of the difference is significant at the 95% confidence level.

DISCUSSION

The clinical and histological manifestations of this neoplastic disease are similar to those described by investigators working with various flatfish in the Puget Sound area (*3–5*). The incidence of the disease in the latter area was reported by Pacis (*9*) and Good (*10*) to be 4.5–4.8%. San Francisco Bay has a significantly higher incidence of the disease. This relatively high incidence is apparently not a new phenomenon, having been reported as about 15% in 1922. A most noticeable difference between the disease in San Francisco Bay and Puget Sound is the species-specificity observed in the former area while in the latter area many species of flatfish are involved. The disease was never seen in any of the members of the other approximately seven species of flatfish taken in the Bay. A very interesting observation, and one possibly related to the occurrence of this disease in only one species, was the occurrence of a monogene trematode (*Gyrodactilus* sp.) on the English sole. This trematode is an ectoparasite and is species-specific. The relationship of this animal to the disease is currently under study by our group.

The distribution of tumors on the surface of the English sole appears to be random, whereas in flatfish taken from Puget Sound the tumors tend to be on the top side of the animal. This randomness suggests that the tendency toward tumors is initiated before metamorphosis takes place. The pigmentation of a tumor correlates with location on the fish, because the top side of the fish is naturally pigmented and tumors on that surface assume the pigmentation. As the tumor becomes larger, it usually becomes more elevated and cauliflower-like, which would explain the correlation that exists between these descriptions. The correlation between texture and pigmentation is unexplained.

The fish length distribution of all the English sole taken during the study indicates that no significant difference exists between the distribution of tumorous and nontumorous fish. This suggests that the disease had no adverse effect on the fish involved. If the disease were lethal, one might expect that the distribution of tumorous fish would be skewed to the left (*see* text-fig. 3). Since dead fish are not captured, differences in death rate between the two groups cannot be measured directly. No tumors are seen in fish much longer than 200 mm, and tumors are not seen with any significant frequency in fish above 150 mm. This and the fact that no tumorous English sole have been reported in the commercial catches off San Francisco suggest that the tumors regress. No debilitation of diseased fish is apparent since the weight-length relationship is identical for both groups.

Most fish have one tumor and those with more than one are of a decreasing frequency. Regardless of the number of tumors occurring per fish, the size frequency distributions are similar (text-fig. 4). This would suggest that the predisposing factors to having tumors occur early in life and do not increase as the fish grows older. In the laboratory, we have seen the spontaneous occurrence, in a matter of days, of up to 3 tumors on fish less than 50 mm long.

Except for the presence of tumors, it had been, up to this point, impossible to detect any noticeable differences between normal and abnormal fish. Further analysis of the data indicated that a significant difference in the manner of growth existed between these groups. During the first year of life, these fish grow to about the same standard length (the asymptotic value A in table 3) but the tumorous fish appear to be born later in the year and grow faster. These curves can be extrapolated to show that the difference in birth time may be more than 2 months. Growth can be considered as a parameter to differentiate subpopulations (*11*); thus, these data may be indicative of such a subgroup existing among the English sole of San Francisco Bay which is particularly susceptible to this tumorous disease.

There is a seasonal effect on tumor incidence that is seen throughout San Francisco Bay, with the highest frequency occurring between May and September. More important is that, regardless of time of year, the incidence of tumorous fish is much higher in the North Bay than in the South Bay. For the whole year, the incidence in the north is about twice that in the south. The general differences between the two areas are: 1) on the average, the north portion is much shallower than the south; 2) there is more influence in the north of the large fresh water inflow from the Sacramento and San Joaquin Rivers; and 3) the north is more directly influenced by industrial waste discharge, particularly petrochemical.

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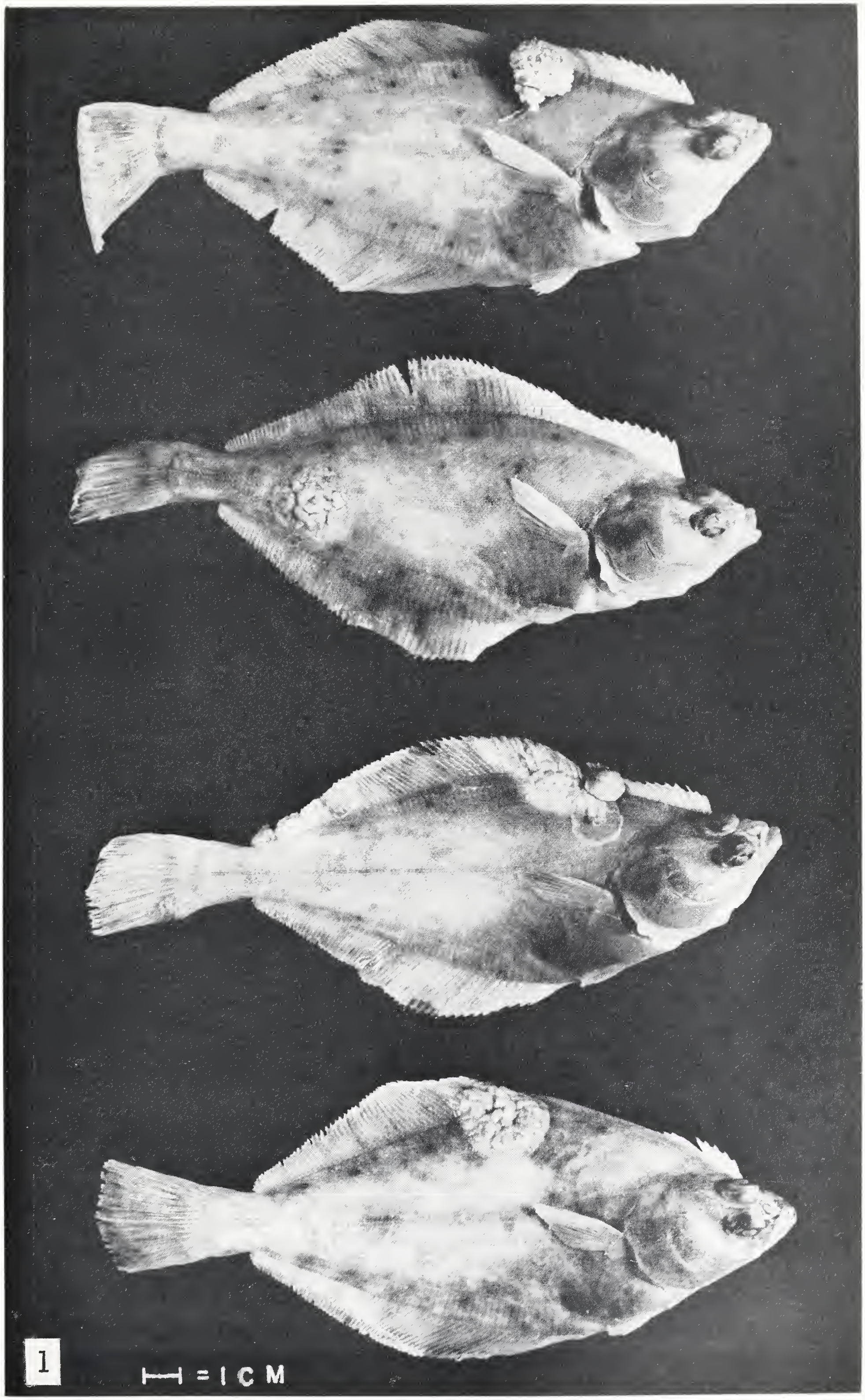

FIGURE 1.—Composite view of four English sole with advanced epidermal tumors.

Papillomas in the Atlantic Eel, *Anguilla vulgaris*

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SUMMARY

"Cauliflower disease," a papillomatous growth on the skin of eels *(Anguilla vulgaris)*, appears to have spread as an epidemic from the Baltic waters to northwestern Europe between 1944 and 1956. About 3% of eels from either fresh or sea water are affected by papillomas situated mostly on the snout. The growths are basically composed of proliferation of skin epithelium. Experimental work until now has not disclosed the cause of this disease. Electron microscopic examination has revealed virus-like particles in the tumors. Monolayer cultures of human and eel cells, when incubated with a suspension of these virus-like particles, showed cytopathogenic effects. Transplantation experiments have been unsuccessful; neither a cell-free filtrate nor small pieces of tumor induced papillomas in healthy eels.—Nat Cancer Inst Monogr 31: 187–193, 1969.

THE "CAULIFLOWER DISEASE," a papillomatous growth of the skin of eels (*Anguilla vulgaris*), appears to have spread as an epidemic from the Baltic waters to northwestern Europe between 1944 and 1956. Some fishermen stated that they observed this disease before 1944, but so rarely that it had aroused no attention. Both Amlacher (*1*) and Mann (*2*) reported that the disease was known long before 1944 in the coastal waters of Germany. We cannot assess whether their observations are really related to the cauliflower disease. It is known, for instance, that eels, affected by the very common "red disease," may have large cystlike tumors on their abdomen and sides; also "pox disease" gives epithelial growths consisting of a hyperplasia of epidermis. The corium remains unchanged, and there, no papillae are formed. This all seems to indicate that the cauliflower disease, even if it occurred before 1944, was, at any rate, very rare.

But in 1946 Danish fishermen complained that the disease was so frequent in some areas that it was disastrous to the fishery, since the affected eels could not be sold.

According to Jensen (*3*) the distribution of the disease would point to a "chief hotbed" of the cauliflower disease somewhere in the eastern Baltic. The comparatively frequent occurrence of diseased eels in Poland just after

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World War II shows that one of the hotbeds may be situated there. Unfortunately, political conditions make it impossible to obtain information about the distribution of the disease along the eastern Baltic.

Until 1949 the cauliflower disease was almost unknown in German waters, but in 1950 it appeared along the eastern coast of Germany, and since 1953 diseased eels have been found all along the North Sea coast of Germany. In some cases 15% of all eels caught either in sea water or in fresh water had tumors.

Since 1956 this disease has occurred in Dutch waters, and now it has spread to Belgium.

The occurrence of the disease in eels shows remarkable annual and seasonal fluctuations: In Dutch waters in autumn, tumors were observed in 5% of the eels, while in springtime they were seldom seen.

However, these data are based chiefly on information gathered from fishermen whose statements were difficult to check. Furthermore, the initial stage of the tumor could have easily been overlooked, being more or less the size of a pin's head.

The facts that these tumors did not seem to have been observed until 1944, that the disease since then has been found frequently during certain periods of the year, and that it has spread geographically suggests an infectious disease, the contagium of which is unknown.

MACROSCOPIC AND MICROSCOPIC DATA

The tumors vary in size and extent. They are mostly situated at the snout (figs. 1 and 2). But sometimes they occur on more caudal parts of the body; in most of these cases small growths are found on the tail or fins.

Although they vary in size or location, all are of the same type, belonging to the benign epithelial papillomas. They are connected to the skin by a stalk (fig. 3); there is no invasion of tumor tissue into the deeper layers, nor are tumors observed in the visceral organs.

The structure of this papilloma consists basically of tissues of epithelial origin, and in all the specimens examined the histologic picture was very much the same. We can distinguish three types of cells in the tumor constituting an important part of the normal epidermis (fig. 4): 1) basal cells, containing chromatin, lining a very scant connective tissue; 2) epithelial cells, less rich in chromatin, irregularly disposed; and 3) mucus-containing cells.

The tumor's surface is slightly rough; it has narrow clefts and the lobate structure is composed of large, proliferated epithelial cells. During the growth of the tumor, these folds come into contact with each other, become fused, and form the "inside" of the tumor (fig. 5).

In the connective tissue there are often capillaries and deposits of pigment. The quantity of this pigment varies from case to case, in accord with the macroscopic appearance of the tumors, whose color can vary from red to gray.

Even in the largest tumors no necrotic processes were observed. Nearly no mitoses were found. With a few exceptions, the tumor did not change much in size during the observation time of 6 months. Although this disease does not cause the immediate death of the eels, the eels' general condition will deteriorate and their viability will decrease gradually because of secondary effects, such as obstruction of the mouth or nose. Comparing the average weight of normal and diseased eels, Lühmann and Mann (*4*) found that the total body weight, as well as the weight of the liver and intestines, was decreased in the diseased eels. Conceivably these tumors interfere with feeding.

POSSIBLE ETIOLOGY

Apart from pathogenic organisms or infectious agents such as a virus, other environmental conditions like temperature, salinity, and oxygen tension may play a role in the expression of the disease. When the eels dig into the sea floor, carcinogenic substances or other chemicals, or mechanical actions on the skin, may alone, or in combination with the above mentioned possible factors, give rise to tumors.

Although an eel can be a host for many parasites of different species, no correlation with the occurrence of tumors was ever proved. Trypanosomidae, known as virus transmitters, are frequently found in the blood.

The well-known papillomas of aquatic animals are found in oysters (*Crassostrea commercialis*), in flounders of several species (*Parophrys vetulus*, *Hippoglossoides elassodon*, *Glyptocephalus zachirus*, *Psettichthys melanostictus*, *Limanda herzensteini*, and *Hippoglossoides dubius*), in croakers (*Genyonemus lineatus*), in bullheads (*Ictalurus nebulosus*), in the flathead sole, and in the Atlantic eel. All these animals live at the bottom of the sea, at least for certain periods. Probably the papillomas in all these aquatic species are related.

EXPERIMENTAL WORK

Transmission experiments have so far been unsuccessful: Neither the injection of a cell-free filtrate nor the implantation of small pieces of tumor induced papillomas in healthy eels. Work is in progress along this and other lines.

That virus-like particles have been revealed in the tumors by electron microscopic examination justified the experiments to obtain some evidence of a viral etiology of this disease. Monolayer cultures of a human cell line (HeLa) and primary eel tissue (gonads) were incubated with suspensions of these virus-like particles at different concentrations. A cytopathogenic effect on the cells was present in many cases. This seems to point to the existence of a virus in the tumor, though up to now there are no indications to conclude that this virus causes the unbalanced outgrowth of skin epithelium. Microscopic examinations revealed no cellular inclusion bodies. Experimental work until now has not disclosed the cause of the papillomas.

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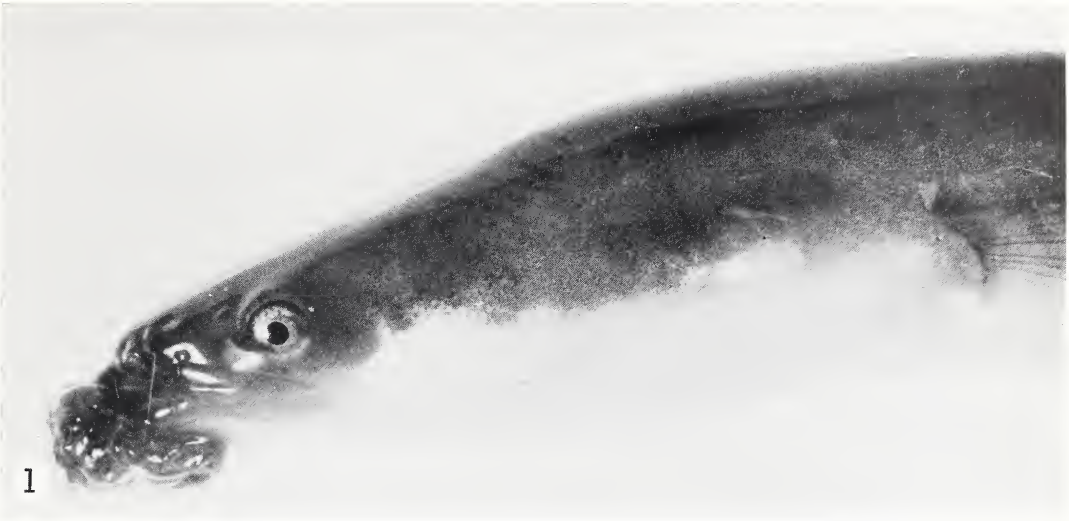

Figure 1.—Eel with tumor on lower jaw. Three times its natural size.

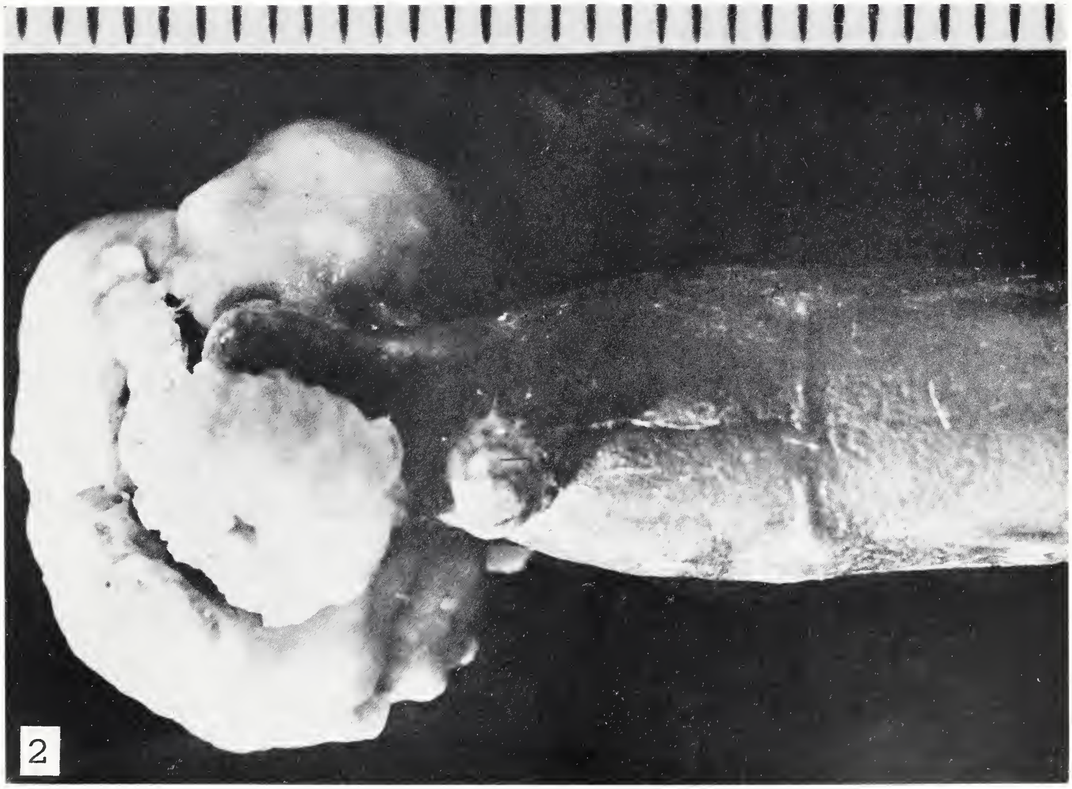

Figure 2.—Gross appearance of tumorous growth on upper and lower jaw; the left eye is covered by a smaller tumor. Viewed from above; five times its natural size.

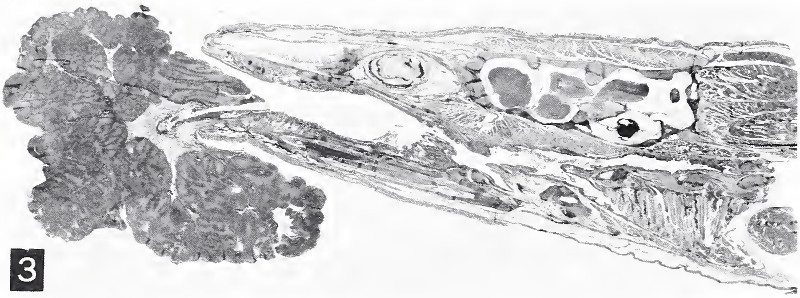

FIGURE 3.—Histologic section of head of an eel with a cauliflower-like tumor on lower jaw. Three times its natural size.

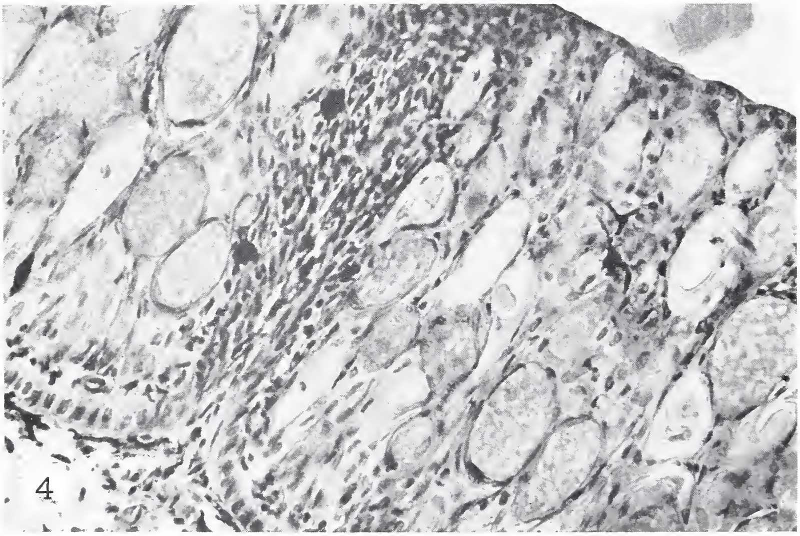

FIGURE 4.—Section of normal eel skin, composed of basal, epithelial, and mucus-containing cells. The condensed cells belong to a chemoreceptor. × 500

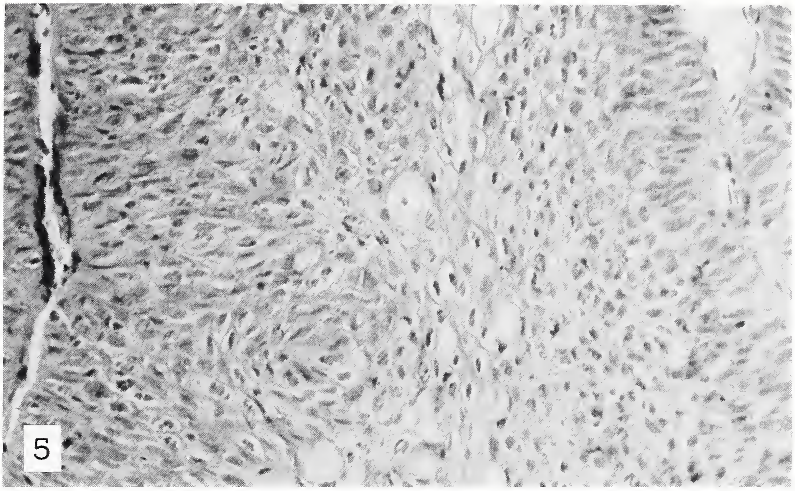

FIGURE 5.—Section of tumor tissue composed of mucous cells, limited on both sides by abundantly growing epithelial cells and basal cells. The delicate streaks of connective tissue show blood elements and pigment. × 500

Virus Associated With Epidermal Hyperplasia in Fish[1]

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SUMMARY

Among walleyes, *Stizostedion vitreum*, in Lake Oneida, New York, three virus-associated skin lesions were found. Besides the widespread lymphocystis disease (fibroblast hypertrophy, with 200 mμ icosahedral virus particles in the cytoplasm) and a locally common dermal sarcoma with 100 mμ surface-budded virus particles, there was an epidermal hyperplasia. This third disease appeared as broad, sharply delimited plaques of thickened epidermis, several cm in diameter, seldom necrotic. Light microscopy showed little alteration except hyperplasia. Electron microscopy of epidermal cells showed much folding of cell surface, with many microvilli in enlarged intercellular spaces. In these spaces there were many 80 mμ spherical virus particles with dense nucleoids. A few were seen budding from the cell surface, the cell membrane continuous with the viral envelope. Intracellular precursors have not been recognized. This epidermal virus is quite different from that shown by Schubert in carp-pox. Despite structural similarity between this virus and that of the walleye sarcoma, they differ in size and in tissue specificity. Though the two types of lesion are common in the same population, they are not often found on the same fish, and the lesions of epidermis and dermis are almost never confluent.—Nat Cancer Inst Monogr 31: 195–207, 1969.

AMONG WALLEYES (pike perch, *Stizostedion vitreum*) in Lake Oneida, New York, there are three fairly common virus-associated skin lesions: *a*) the well-known lymphocystis disease, *b*) a dermal sarcoma or fibroma, and *c*) the epidermal hyperplasia reported here. Though the epidemiological patterns of the two latter diseases are not well known, electron microscopy indicates strongly that the associated viruses, presumably causal, are different for the three types of lesion.

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[2] With the technical assistance of Douglas C. Gillespie; for advice and help in the gathering of material I wish to thank various members of the New York State Conservation Department, especially John Greeley, Kenneth B. Nichols, and Robert Griffiths; also the late Frank Youmans and Norman Youmans of the Constantia Hatchery, and John L. Forney of the Cornell Biological Field Station, Bridgeport, N.Y.

LYMPHOCYSTIS DISEASE

This disease, well-known in a variety of freshwater and marine fishes, involves enormous hypertrophy, but no multiplication of the virus-infected connective tissue cells; *i.e.*, the dermal warts or lymphocystis "tumors," though sometimes massive, are not neoplastic (*1*, *2*). An infected cell becomes gigantic, with microvillous surface, and is encapsulated. Its nucleus is proportionately enlarged, with elaborately folded nuclear envelope. There is a high degree of polyploidy and nucleoli are enormous. At maturity the walleye lymphocystis cells may be 0.5–0.7 mm in diameter, and the cytoplasm has an elaborate lacework of DNA or DN-protein inclusion substance. At the surfaces or in the interstices of this inclusion are vast numbers of virions, many millions per cell. The capsids are about 200 mμ in diameter as measured in methacrylate sections. Virions are not found in the nucleus, and probably do not leave the host cell until its death or accidental disruption (*3–5*).

Development of walleye lymphocystis cells was well described by Weissenberg (*6*), but the later growth period is not so well known. Development is slow and surely takes months to maturity of the cells. In Lake Oneida the cycle may possibly take a year from infection during the hatchery-crowded spawning in April to the massive warts of the next April's spawning.

Similar, perhaps identical, viruses affect other perches here and in Europe. Similar but recognizably distinct lymphocystis diseases affect sunfish and bass, *Centrarchidae*, and various marine flatfish, *Pleuronectidae*, as well as many other species for which reports are less extensive (*1*, *7–9*).

The various lymphocystis viruses are like poxvirus of higher vertebrates in their cytoplasmic site of formation and DNA inclusion. However, structurally and in method of formation they more closely resemble the insect iridescent viruses of Tipula (TIV), Sericesthis (SIV), Chilo (CIV), and mosquito (MIV) and their relatives. However, the extent and slowness of host cell growth, and the multiplicity of virions in a mature host cell, are unique features of the group of lymphocystis diseases.

DERMAL SARCOMA OF STIZOSTEDION

The dermal sarcoma of walleyes has been only briefly described [(*10–12*), and shown in Walker's electron micrograph in (*7*)]. It has been found in Lake Oneida, New York, for many years. We also have specimens from Lakes Champlain, Erie, and Huron, and have had unpublished reports from elsewhere. Macroscopically, confusion is easy between these and lymphocystis warts, and the sarcoma may well have been ignored because of this superficial gross resemblance. Even a hand lens makes the distinction clear. The sarcomatous nodules show no giant cells; they are generally more smoothly hemispheric than lymphocystis warts; they are fine textured, variably firm, and variable in vascularization, from pink to white. The nodules are often multiple and sometimes randomly scattered, occasionally in patches of close array, with small nodules centered on scale tips as though individually initiated by a common abrasion and infection. The intact epidermis over large dermal nodules may be either stretched thin or slightly hyperplastic.

Microscopically, there is a range of texture from highly cellular, often chaotically arranged sarcoma, to densely fibrous, tightly whorled, hard fibroma. Electron microscopy shows cells of various form, but generally they are cells with extensive pseudopodial processes reaching into interstitial space. Intercellular virus particles, about 100 $m\mu$ in diameter, are fairly common, though seldom in masses. A few are seen budding from the cell surface; the viral envelope is continuous with the cell membrane, though somewhat thickened. A dense hemispheric layer lies below this half-formed envelope, becoming spherical as the stalk elongates, and later becoming a dense nucleoid. No other viral precursors are recognized in the electron micrographs. Virions are not seen within the cells except occasionally in vesicles which may represent infoldings of cell surface.

We have no adequate experimental or epidemiological evidence for the time course of the disease or its seasonal incidence. During the April spawning run at the Constantia Hatchery on Lake Oneida, a rough estimate suggests that up to 5% of mature walleyes show sarcomatous nodules. This is not unlike the incidence of lymphocystis warts. Both types of lesion may be multiple; both are seen in males and females. The relative frequency of the two diseases seems to fluctuate in successive years. A few walleyes have both types of wart—rather more than would be suggested by the chances of 5% × 5%. This might be interpreted as special vulnerability to both types of virus on the part of fish with extensive abrasions (or with heavy infestation by a hypothetical common vector, for which we have no evidence in either disease).

EPIDERMAL HYPERPLASIA IN STIZOSTEDION

The virus-associated epidermal hyperplasia of walleyes, here described, is known so far only in Lake Oneida during the April spawning run. There has not been adequate sampling at other seasons or places. Somewhat similar lesions in other fish have not been examined for virus, or have shown virus of different type and of doubtful etiological significance, or have shown no virus (*13*). One electron micrograph from the present study was published in Wolf's review (*7*).

The lesions appear as broad, flat, sharply delimited plaques of thickened epidermis, sometimes up to several cm in diameter. They may be anywhere on the head, body, or fins—perhaps most commonly on the tail fin. They are often multiple (fig. 1). There is no visible effect on the dermis, and even in the thinner fin webbing a lesion on one surface seems to have no correlation with possible lesions on the opposite surface (figs. 2 and 3). The edge of the plaque may be very sharply defined (fig. 3), the epidermis showing a precipitous rise to 0.3–0.5 mm and 30–50 cell layers, from perhaps a fifth of that in the normal area; and this increase occurs within a fraction of a millimeter. Gland cells may still be present (figs. 2, 4, 7); more often not (figs. 4–6 and 9).

The grayish appearance of these patches, especially over dark, pigmented dermis, is merely the result of thickening. The grayness has often been mistaken for fungus infection, but careful inspection shows no similarity. The surface is firm and smooth, and microscopically the lesions are remarkably free of bacteria or fungi. Indeed, aside from increase in number of cell layers, there is little noteworthy about the epidermis, except rather more than usual cellular infiltration into the layers close above the basement membrane and germinal layer (figs. 2, 4, 7, and 8). As seen in our limited collection (middle of the day, in the April spawning run only), there is very little mitosis, even in the germinal layer.

The germinal layer is coherent and rests smoothly on the basement membrane (figs. 6 and 8). Throughout the thickened epidermis there are well-formed desmosomal contacts, but the amount of intercellular space varies (figs. 5, 9–11). Many of the pseudopodial processes seen in the wider spaces may be residues of separated cell interfoldings such as are seen in the closely apposed cells of figure 10. The few surface layers of squamous cells are pulling apart (*top* of fig. 5), and electron microscopy shows that in these decadent, dense cells the processes have been preserved.

Electron microscopy at low magnification shows that most cells of the intermediate layer look relatively normal (fig. 10). The nucleus is round or oval, with only slight surface indentations, and the nucleolus is well formed, often lying against the nuclear membrane. There is usually a definite perinuclear zone of mitochondria and endoplasmic reticular vesicles, with or without ribosomes. The latter are scarce elsewhere in the cell. Many cytoplasmic filaments converge on the desmosomes (fig. 11).

The special characteristic of this hyperplastic epidermis at the electron microscopic scale is the fairly consistent presence of intercellular virus particles at every level. They are visible even in figure 10, but at higher magnification they are more striking (figs. 11–16). Mature particles are not found in nucleus or cytoplasm; the "vacuole" with a virus particle in the center of figure 15 is presumably an infolding of cell surface.

Such particles are not seen in the subjacent dermis. As for normal epidermis of the same fish, electron microscopic controls have been inadequate, but negative for virus. Likewise in the epidermis over lymphocystis warts and over dermal sarcomas, such particles are not seen. We have not done sufficiently precise sampling for electron microscopy to define the distribution of virus relative to the sharp edge of the lesion. Not every electron microscopic section of hyperplastic epidermis shows the virus, but most do in samples taken each April from 1964 to 1968.

A very few half-formed particles are seen budding from the cell surface (*arrows* in figs. 12–16). The viral envelope is continuous with the cell membrane, though locally thickened. A dense, hemispheric layer beneath the envelope (fig. 14) is completed into a sphere as the stalk is constricted (fig. 16). Eventually this inner "doughnut" condenses into a nucleoid or nucleocapsid. There is no evidence of the nucleic acid type of this virus, though the developmental pattern is generally associated with RNA viruses.

There is some variation in size and texture of virus particles. The average diameter is about 80 mμ, measured at the densest line of the envelope. Beyond this there seems to be an indefinite halo. The smoothest contours are seen on newly budding particles and in others with a similar central pale area. As the nucleoid condenses, the surrounding space increases, and the envelope may be less regular in form.

These thickened epidermal patches have been seen for many years at the April spawning run at the Constantia Hatchery, but were largely ignored. In early years the more dramatic lymphocystis warts and sarcomas were the center of attention during a busy day of tissue sampling and fixation. Since 1964, repeated check has shown the consistency of the disease at that place and season, and electron microscopy showed the consistency of the associated virus. Incidence is not unlike that of the two other viral skin lesions in these breeding walleyes: about 5% (2%–10%?). As with the other two diseases, double infection is not rare. A few fish may have this epidermal lesion as well as either lymphocystis warts or sarcoma. There is no tendency for topological coincidence of the lesions: We have no record of such altered epidermis directly overlying a dermal wart.

The similarity of form and developmental pattern between this virus and that associated with the sarcoma might have suggested identity. However, aside from the difference in size (which was at first ignored; about 80 mμ here, compared with 100 mμ for the sarcoma), there seems to be fairly strict host-tissue specificity. The two viruses are apparently restricted to epidermis or to connective tissue, respectively. It is planned to investigate this by tissue culture methods.

The possible relationship of this disease to various epidermal hyperplasias in other fish is quite doubtful. Walker (*13*) discusses two apparently nonviral epidermal hyperplasias; that of the yellow perch is grossly similar to this; the dramatic but transient lesions of bluegills look very different. None of these attain the massively folded papillomatous condition described in various species. Though fish-pox, and especially carp-pox, often show heavily papillomatous growth, some reported lesions are grossly similar to this epidermal hyperplasia of the walleye. There have been reports of virus associated with carp-pox, but they have been contradictory (*14–16*). The most convincing electron micrographs are those of Schubert (*16*). However, the virus that he describes in carp-pox is quite unlike this one, being initiated in the nucleus, and becoming mature before emerging from the cell. Unless "fish-pox" is to become a merely general term, we cannot suggest that this walleye disease fits it.

We have no knowledge of the time course of the disease or of the method of transmission. Indeed we have no experimental proof that this new virus causes the epidermal hyperplasia, but the high correlation strongly suggests it. Studies are needed, but not presently planned, as to whether these lesions are commoner at the breeding season or whether they regress spontaneously. It is improbable that they are initial stages of frank neoplasia, for neither papilloma nor carcinoma of the skin has been recorded for walleyes in Lake Oneida. In conclusion, it seems that we have here in *Stizostedion* a third, relatively bland, virus-associated skin disease.

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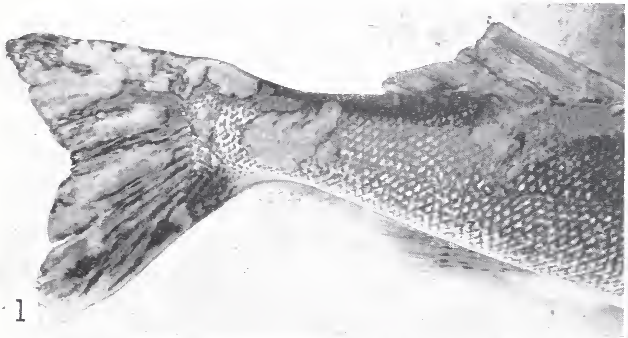
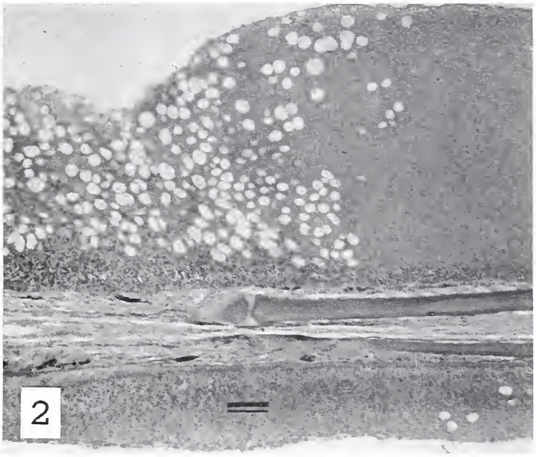
PLATES

All figures are of the walleye, ***Stizostedion vitreum.***





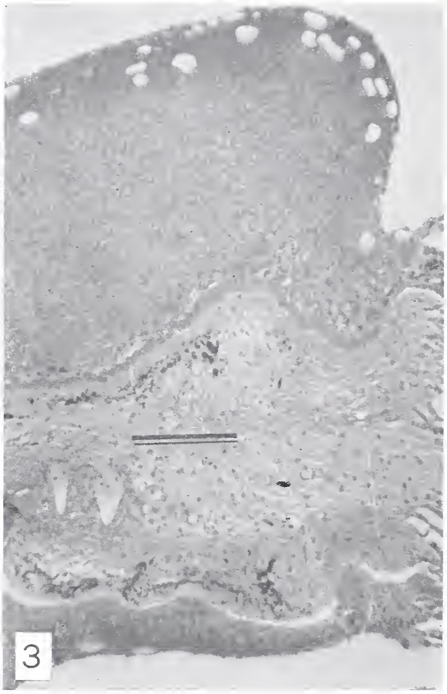


FIGURE 1.—Epidermal hyperplastic lesions on fins, body, and tail peduncle of a mature male.

FIGURE 2.—Section of tail fin. Connective tissue is normal. One epidermal surface shows slight hyperplasia. The other is extremely hyperplastic. One segment has many gland cells; another has none. × 54 (In figs. 2–5 the *double bar* represents 100 mμ.)

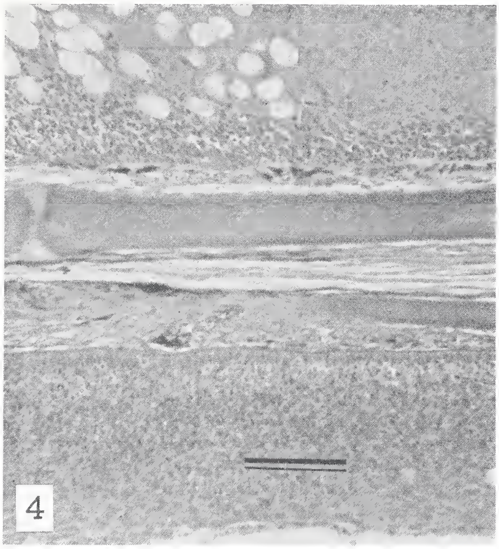
FIGURE 3.—Normal epidermis is *below;* sharp boundary between hyperplastic and normal epidermis is *above.* × 136

FIGURE 4.—Detail from figure 2. Cellular infiltration above germinal layer is greater in segment with gland cells. × 136

FIGURE 5.—Distal layers of hyperplastic epidermis without gland cells. There is some intercellular space. Only the most superficial layers show squamous decadence. × 540

FIGURE 6.—Proximal quarter of hyperplastic epidermis with minimal cellular infiltration. × 540

FIGURE 7.—Detail from figure 4. × 540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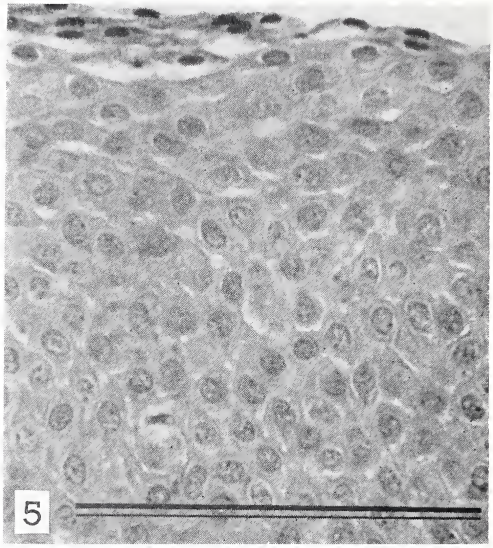
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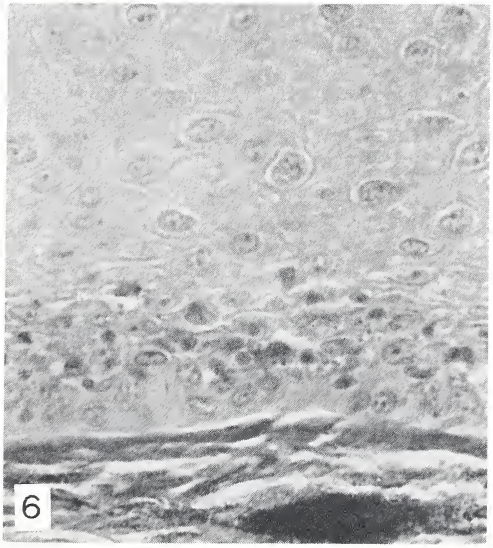
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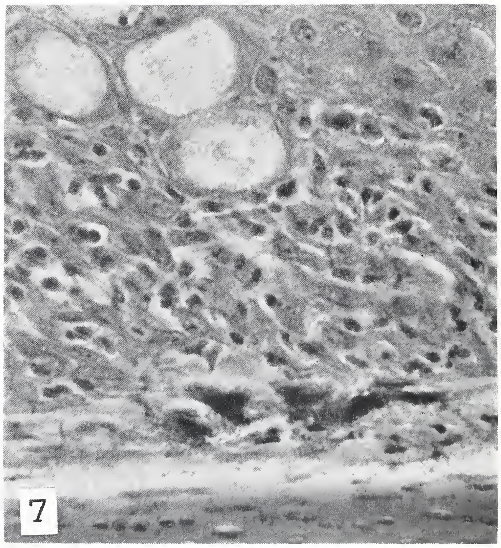
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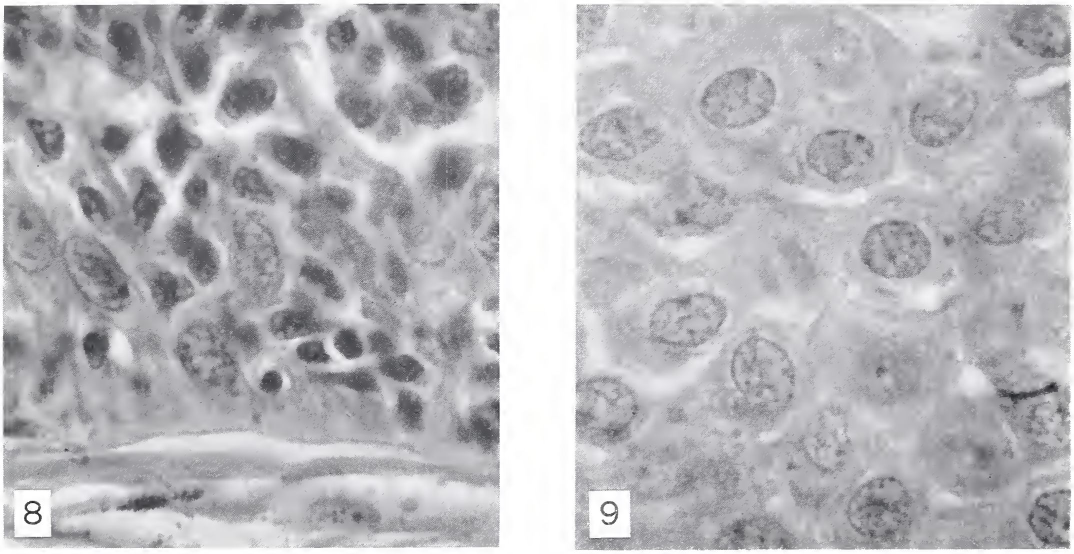
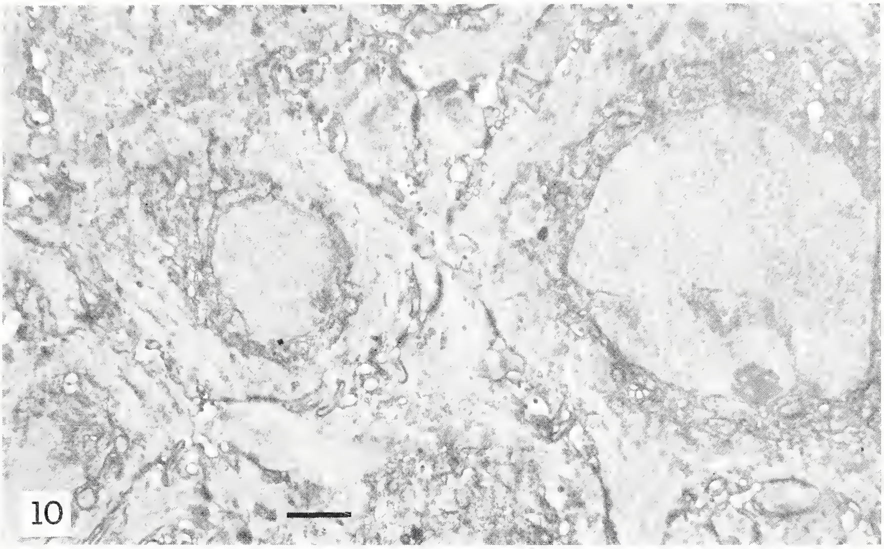


FIGURES 8 AND 9.—Higher magnification of areas like those in figures 7 and 5: basal infiltration and distal layers, respectively. × 1360

FIGURE 10.—Electron micrograph (low magnification) showing relatively normal cells like those in figure 9. There are intercellular virus particles. × 8500 (In figs. 10–16 the *single bar* represents a micron.)

FIGURE 11.—Intercelluar virus particles. Cytoplasmic filaments diverge from desmosomes. × 33,600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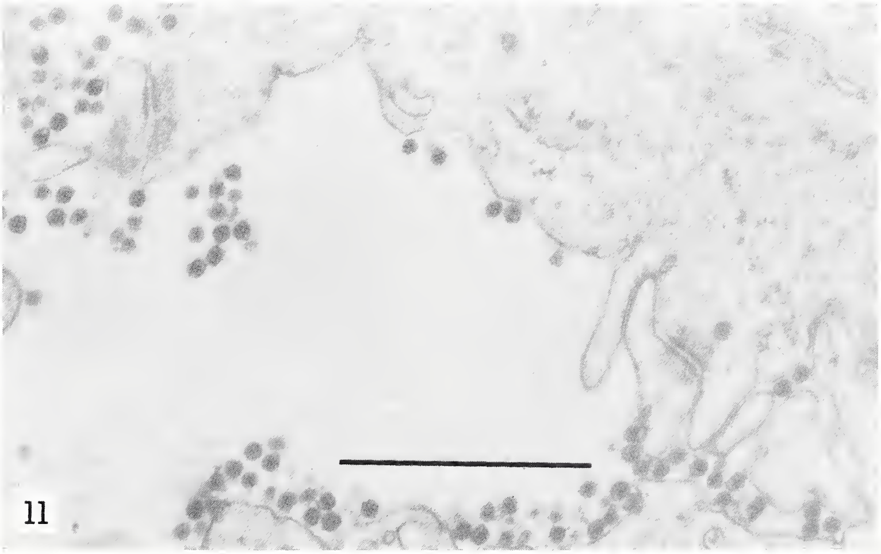
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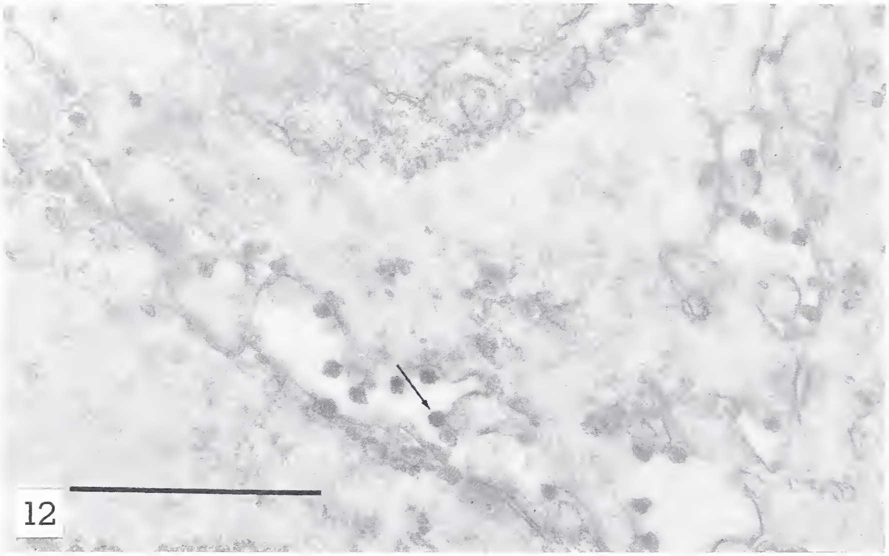
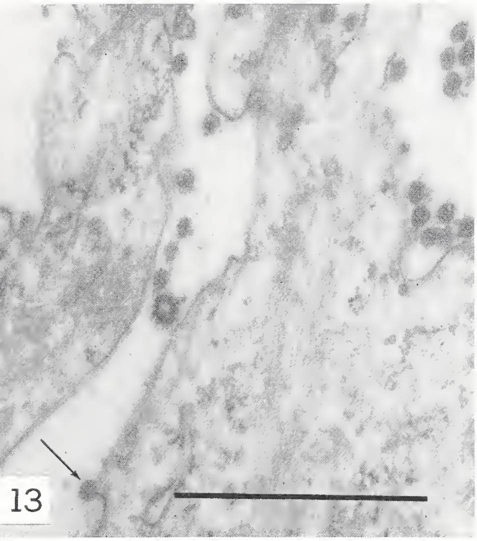


FIGURE 12.—Intercellular virus particles. Cytoplasmic filaments diverge from desmosomes. *Arrow* points to a particle budding from cell surface. Cells have interdigitation processes and desmosomes. There is a zone of perinuclear organelles (*top*). × 33,600

FIGURES 13–16.—Details of cell surfaces and intercellular virus. *Arrows* point to budding particles. Figures 13 and 14, × 34,000. Figures 15 and 16, × 51,000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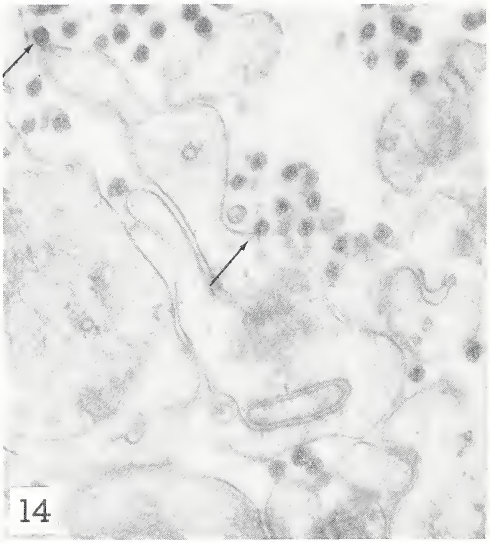
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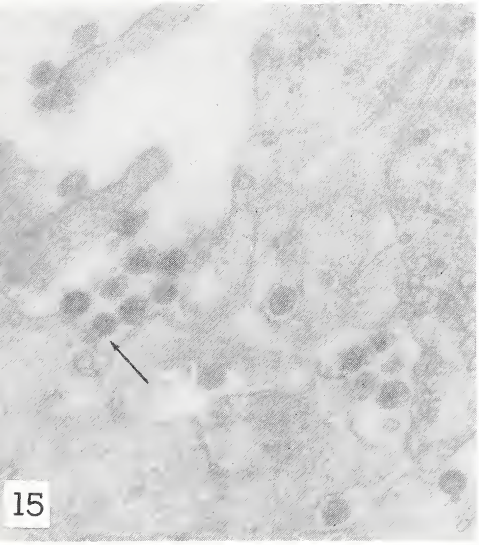
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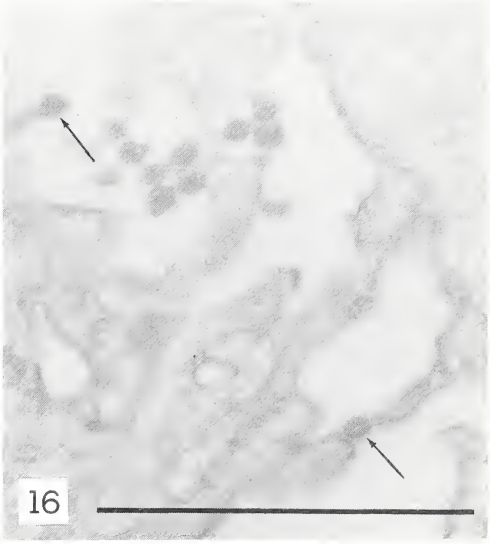
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Epidermal Hyperplasia in Fish: Two Types Without Visible Virus[1]

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Epidermal hyperplastic lesions of walleyes, *Stizostedion vitreum*, are consistently associated with an 80 $m\mu$ surface-budded virus, at least during the April spawning season in Lake Oneida, New York (*1*). The fact that this locally common lesion has been so little noticed, or has been mistaken for a fungus infection, raised the suspicion that the disease might be widespread, perhaps also in other species.

EPIDERMAL HYPERPLASIA IN PERCA

By personal watching and inquiry we found yellow perch, *Perca flavescens*, with lesions similar in appearance to those of walleyes: broad, circumscribed, gray-white thickenings of the epidermis. One specimen was from Lake Oneida. Others, from Cassiday Lake, Michigan, were kindly supplied by James C. Schneider of the Institute for Fisheries Research, Ann Arbor. He wrote that in a January collection, in 1967, similar lesions were found on 14% of perch over 5 inches long, but none on smaller ones. There was significant incidence at other seasons, in other years, and in other Michigan lakes.

Microscopy showed neither bacteria nor fungi. Lesions from three perch were studied by electron miscroscopy after fixation in buffered osmic acid and methacrylate embedding. The hyperplastic epidermis consisted of essentially normal-appearing cells (fig. 1), and no virions were found after prolonged search. This sampling must be considered inadequate, both as to number and season; further investigation is needed.

EPIDERMAL HYPERPLASIA IN LEPOMIS

A very different epidermal hyperplastic lesion was described by Nigrelli (*2*) in bluegills, *Lepomis macrochirus*, and has been repeatedly found in our own laboratory aquaria. The disease may appear as an "epidemic," frequently within a week or two after a new batch of bluegills or other sunfish has been put into a common holding tank. Such fish have come from our local ponds, from a commercial hatchery in Pennsylvania, or from Dr. Harry K. Dupree of the Southeastern Fish Cultural Laboratory in Marion, Alabama. Whether such fish seemed healthy on arrival or were apparently suffering from the effects of delayed shipment, they showed no epidermal lesions on arrival. Within a few days after the first small lesions appeared, many of the fish showed multiple epidermal tabs (fig. 2), often growing one above each scale, but sometimes confluent. These growths were often higher than wide; in extreme cases the fish looks "hairy" or as though fungus-infected. However, the "filaments" are definitely epidermal: not raggedly sloughing, but with extreme localized growth (fig. 3). The affected fish do not eat well, and sometimes there is increased mortality during the epidemic, but generally the epidermal growths regress within 2 weeks and do not recur. Neither bacteria nor fungi seem to be involved.

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[1] Aided by Public Health Service grant CA 05790 from the National Cancer Institute.

[2] With the technical assistance of Douglas C. Gillespie.

Of specimens from several years and seasons studied by electron microscopy, almost all showed no virions either in or between cells. In only one specimen were there very few intercellular "virus-like" granules not surely distinguishable from other small particles. Many of the epidermal cells appeared relatively normal, but consistently there was a fair proportion of cell pathology. There were many decadent cells, phagocytes containing cell debris, and cells with exaggerated myelin figure inclusions (figs. 4 and 5).

Nigrelli (*2*) equated this disease with the fish-pox so often described in Europe. There is recurrent doubt as to the etiology of European fish-pox, especially carp-pox. Nutritional, chemical, hormonal, and stress factors have been suggested, as well as virus as a possible contributing agent. Unfortunately, the various reports of virus (*3–5*) are discordant. The electron micrographs of Schubert (*5*) are elegant and convincing as to the presence of virus in his case, but the etiological role of this virus in carp-pox in general is still uncertain. There is even a difficult question of the proper characterization of fish-pox. The etiology of epidermal hyperplasia of bluegills is as yet no better determined, but extensive sampling for electron microscopy seems to make it improbable that a virus is the primary agent.

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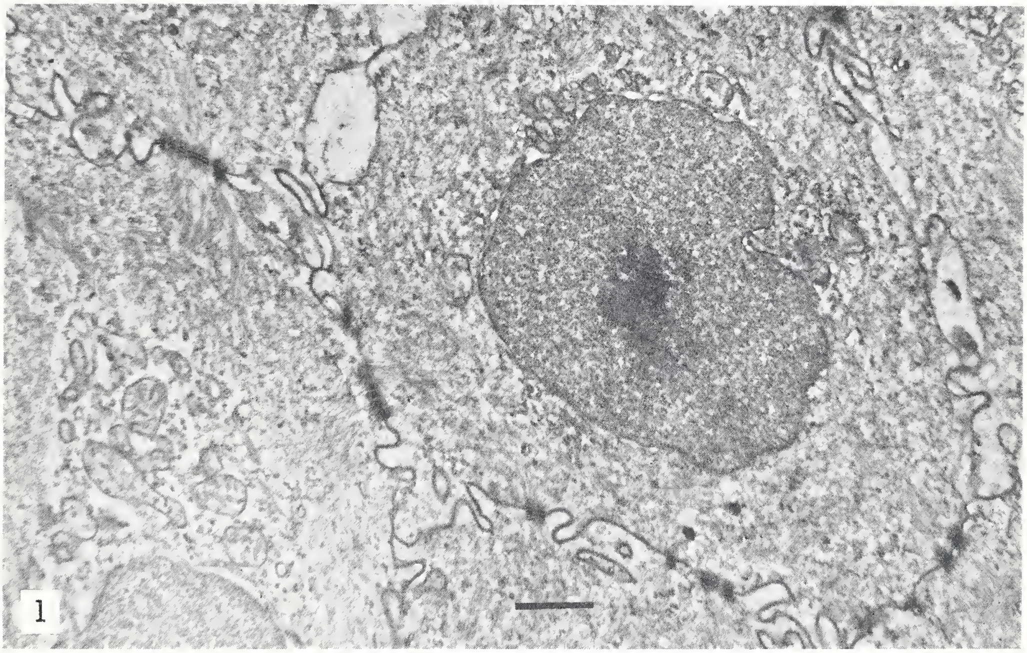


FIGURE 1.—Normal-appearing cells of hyperplastic epidermis of yellow perch. Micron line. × 10,000

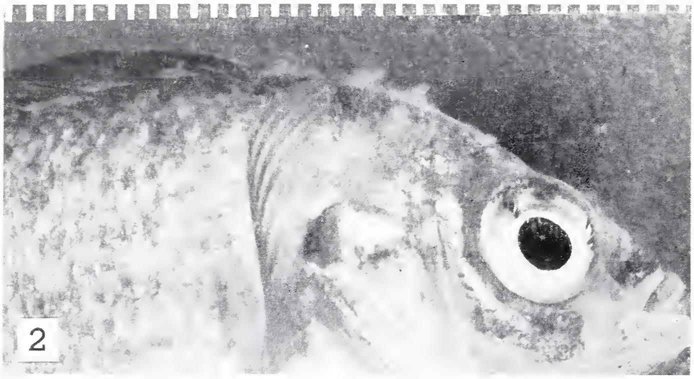


Figure 2.—Bluegill sunfish with hyperplastic epidermal tabs. Millimeter scale.

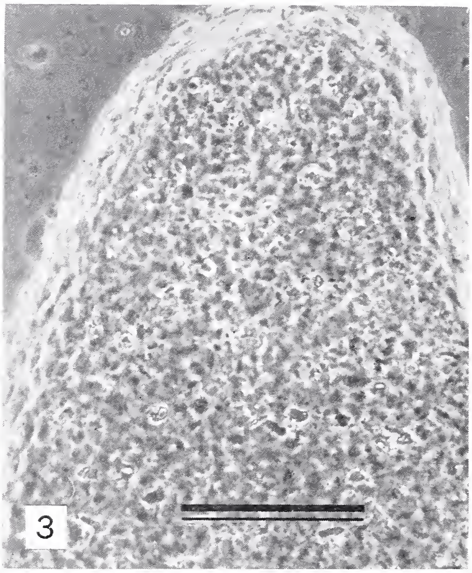
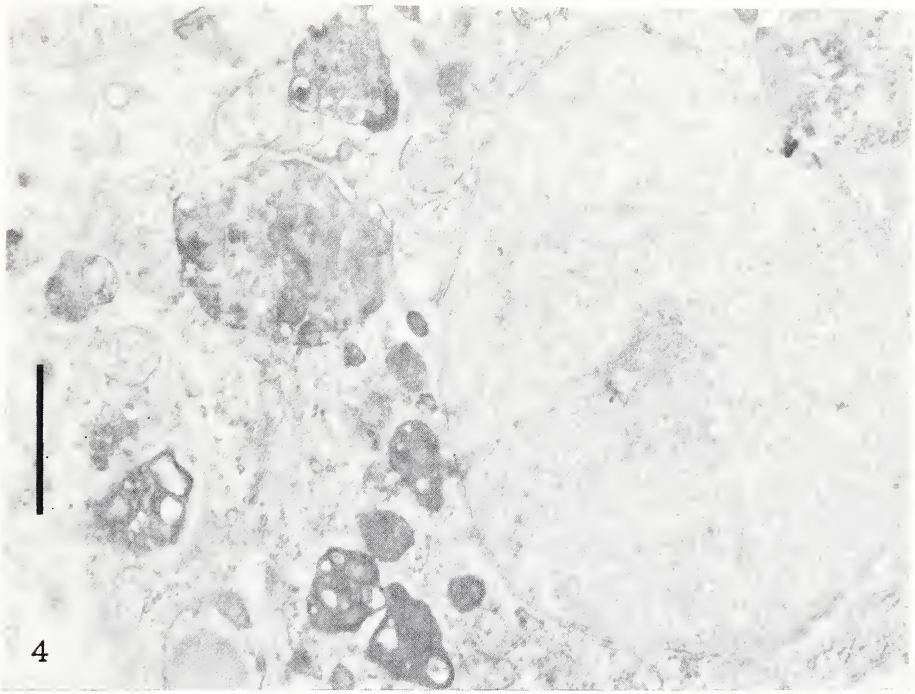
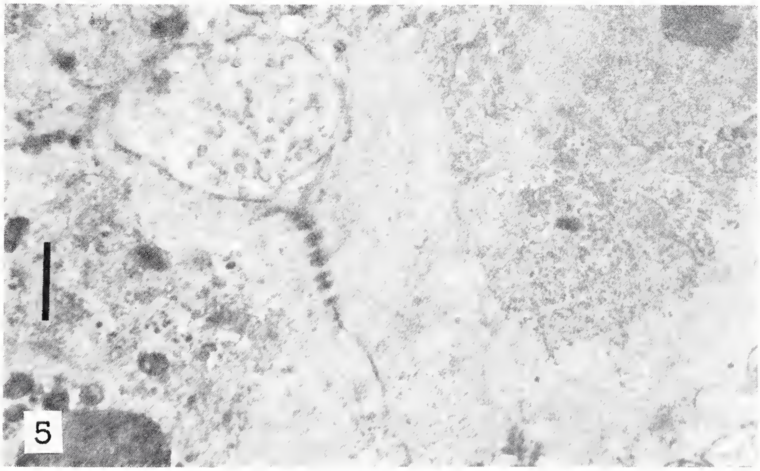


Figure 3.—Tip of epidermal tab from bluegill; fresh, phase contrast. × 240; double line is 100 μ.



IGURE 4.—Cell pathology in hyperplastic epidermis of bluegill: inclusions and myelin figures. Micron line. × 20,000



IGURE 5.—Bluegill: fairly normal cell and adjacent cell with inclusions. Micron line. × 10,000

An Epithelial Papilloma of the Brown Bullhead, *Ictalurus nebulosus*

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An epithelial papilloma (squamous) was observed on a brown bullhead (*Ictalurus nebulosus*) collected from Lake Drummond in the Dismal Swamp area of southeast Virginia. The animal was 28 cm long, weighed approximately 325 g, and appeared to be in good health at the time of collection.

A single large tumor 1 cm high and 2 cm in diameter was located in the middorsal line directly between the eyes. The gross structure of this tumor was similar to that of a common wart, exhibiting a keratinized surface, with the characteristic lobulated appearance of papilloma. Histological structure of this papilloma did not differ significantly from that of previously described epithelial papillomas in fish (*1*). Proliferation of the epithelium with resultant thickening and cyst formation was evident. Supporting the masses of epithelial cells was a delicate arborizing connective tissue stroma. Cellular areas adjacent to the connective tissue stroma tended to be columnar or cuboidal in shape, while those toward the centers of cystic areas were irregular or spindle shaped. Nuclei were prominent, appeared somewhat enlarged, and had conspicuous nucleoli. Within the lumens of the cysts a certain amount of mucus was observed, the product of mucus-secreting cells located in the epidermis.

The tumor was sharply circumscribed and exhibited no invasion of the subjacent tissue. There was no noticeable inflammatory reaction.

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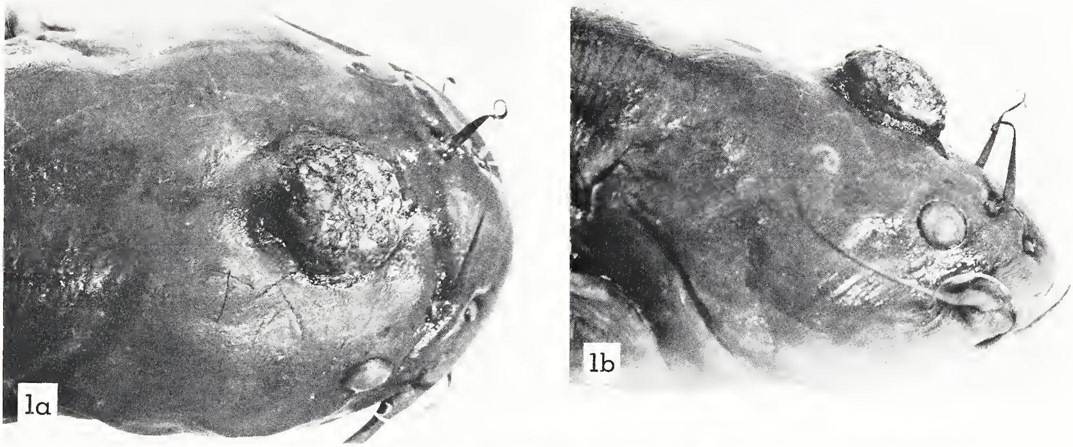

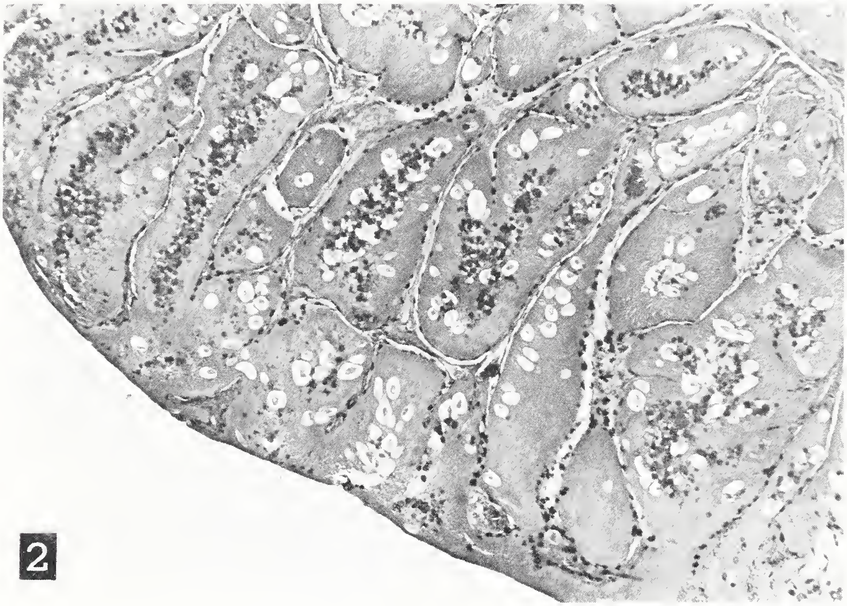

FIGURES 1a AND 1b.—Dorsal and lateral views of papilloma of the brown bullhead, *Ictalurus nebulosus*. Approximately life size.

FIGURE 2.—Low-power photomicrograph of section of papilloma shown in figures 1a and 1b. *Note* connective tissue trabeculae, cyst formation, and mucous secretion into the lumens of certain cysts. × 70

Case Report of a Fibrolipoma on a Rockfish, *Sebastodes diploproa*

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The specimen was taken by one of the authors (G. W. B.) during a survey collecting trip in California coastal waters. The fish was caught at 120 fathoms in the Big Sur area, approximately 120 miles south of San Francisco, and was immediately fixed intact in formalin.

The fish, 25 cm long, had an approximately hemispherical protruberance on the left side in the mid-dorsal region (fig. 1). The distended overlying skin was thin and had lost its scale pattern.

Removal of the anterior half of the mass revealed a bright-yellow cut surface, slightly streaked with pale-gray, linearly raised areas. The mass was very firm, having the consistency of dense fibrous tissue or cartilage. It was attached deeply to the underlying muscle and superficially to the overlying skin, but was sharply circumscribed. The anterior portion was easily shelled out by blunt dissection. The mass, measuring approximately 5.5 × 4.8 × 2.3 cm, extended from about 1 cm ventral to the lateral line to the base of the dorsal fin. There were no metastases and no evidences of other tumors.

Sections were made and stained with Sudan IV, hematoxylin and eosin, by van Gieson's method, and with the periodic acid-Schiff reaction, with and without pretreatment with diastase.

Microscopically (figs. 2 and 3) the tumor was well encapsulated; neither adjacent muscle nor overlying skin was invaded. The tumor was composed of mature fat cells, containing fat in large droplets, generously dispersed among islands and strands of collagenous tissue, except in the peripheral area where the collagenous tissue predominated. There was no association of nerves, vascularization was not prominent, and mitoses were not observed. Based on these characteristics, the lesion was diagnosed as a fibrolipoma.

When autopsied, the specimen was found to be parasitized by *Anisakis* nematodes, free larvae of which were randomly scattered throughout the peritoneal cavity. However, nematodes of this genus are common parasites of marine fish and mammals (*1*), and their presence probably did not influence the development of the fibrolipoma. Other parasites had produced fibrotic cysts which appeared grossly as small whitish protrusions in the spleen. Microscopically, the remains of these parasites were unsymmetrical, acellular, and structureless, possibly immature tapeworm larvae (fig. 4).

Only ten cases of fish lipomas have been reported, each report being based on one specimen and each representing a different species (*2*, *3*). Only two tumors have been reported from rockfish: a fibroma in the body wall near the caudal fin of *Sebastodes inermis* and an osteoma of the dorsal fin of *S. inermis* (*4*).

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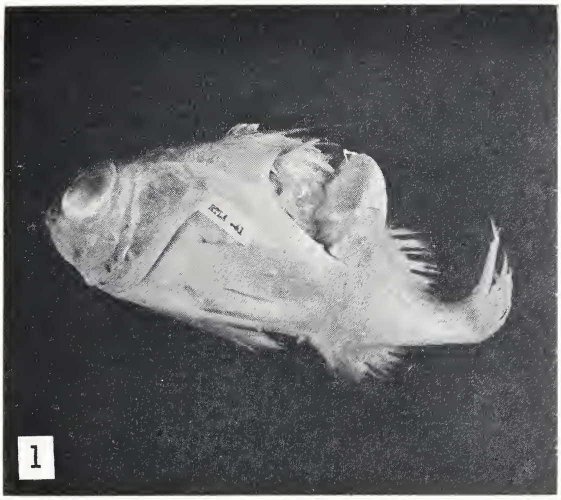

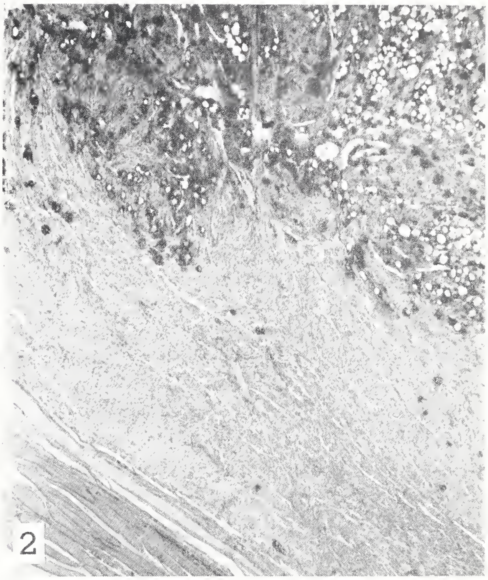

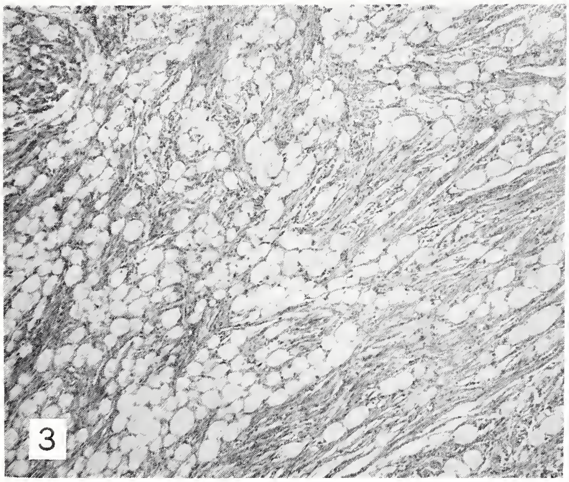

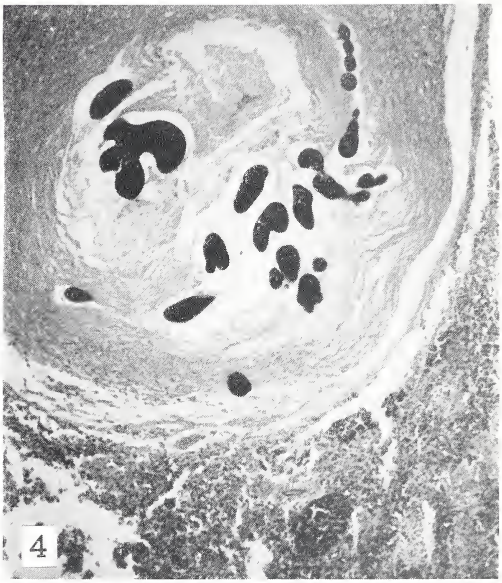

FIGURE 1.—Rockfish with anterior half of tumor removed.

FIGURE 2.—Edge of tumor capsule and underlying muscle in *lower left corner*. Dark bodies are fat droplets stained with Sudan IV. *Note* the peripheral area of the tumor is composed primarily of fibrous tissue and most fat cells occur deeper. × 27

FIGURE 3.—Mature fat cells dispersed among islands and strands of collagenous tissue. × 90

FIGURE 4.—Unsymmetrical, acellular, structureless entities encysted within the spleen. Possibly remains of immature tapeworm larvae. × 70

Biochemical and Morphologic Studies of Lymphocystis Disease[1]

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SUMMARY

Lymphocystis disease is characterized by an enormous cellular hypertrophy with massive accumulation of cytoplasmic RNA and DNA and the formation of a hyaline capsule around the cell membrane. The hypertrophied cells form tumors in the integument of fishes. The causative agent contains DNA and has a cytoplasmic site of replication. The work presented revealed the following: Early in infection of tissue cultures, under conditions of high multiplicity of infection, the cells became altered but the cytopathic change consisted essentially of cell rounding and not of cellular hypertrophy. There were distinct increases in the rates of synthesis of DNA, RNA, and protein, but these macromolecules did not accumulate above the levels in normal cells. It is assumed that the increased rates of synthesis were accompanied by increased catabolism. These findings may reflect the effect of the high multiplicity of infection used, because low inputs of virus in tissue cultures, as well as the natural *in vivo* infection, result in distinct morphologic cellular enlargements and accumulation of nucleic acids.—Nat Cancer Inst Monogr 31: 223–236, 1969.

LYMPHOCYSTIS DISEASE, long recognized in freshwater and marine fishes, is characterized by nonmalignant papilloma-like lesions of the connective tissue, the gross appearance of which is shown in figure 1. The tumors are due to enormous hypertrophy of the host tissue cells under the influence of an infectious agent. Although the etiology was unknown for many years and various parasites had been suggested as causal agents, the concept of a viral causation was proposed as early as 1914 by Weissenberg (*1*). This hypothesis has been confirmed by the recent *in vitro* isolation of the virus from freshwater fishes (*2*) and from marine species (*3*).

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The main histopathologic features of the disease *in vivo* (fig. 2) are intense cellular hypertrophy, extensive cytoplasmic inclusions, and the formation of a hyaline capsule in advanced stages of infection. It is to be emphasized that, however large the tumors, they are not neoplastic; the infected cells do not multiply. The lesions consist only of those cells individually affected and there is negligible hyperplastic reaction of the adjacent tissue. The infected cells grow slowly and reach full size of up to 1500 μ in a few weeks to a year, depending upon the host species and environmental conditions (*4*, *5*).

Reichenow (*6*) reported in 1928 that the cytoplasmic inclusions associated with lymphocystis cells are Feulgen-positive, indicating the presence of deoxyribonucleic acid (DNA). Electron microscope studies of tumor tissue by Walker and Weissenberg (*7*) and of infected tissue culture cells by Zwillenberg and Wolf (*8*) and Midlige and Malsberger (*9*), plus autoradiographs of infected cells *in vitro* (Malsberger, personal communication), have led to the assumption that replication of viral DNA, production of viral protein, and virus maturation are purely cytoplasmic events. Such cytoplasmic replication of DNA-containing viruses has been shown to date in only three other instances: the poxviruses (*10*), the frog viruses recently studied by Granoff *et al.* (*11*, *12*), and, presumably, the iridescent virus group (*13*).

Most of the studies on lymphocystis disease and its causative virus have been with isolates from freshwater species, but marine fishes—snappers and angelfish[4]—with lesions characteristic of the disease were observed in the Florida Straits. From each of several such tumors a virus has been isolated in tissue culture cells of marine fish origin by Sigel *et al.* (*3*), and one such isolate is currently in its 75th serial passage. The *in vitro* cytopathology, as *in vivo*, is manifested in cellular hypertrophy and the occurrence of basophilic cytoplasmic inclusions (figs. 3 and 4). In addition, the infected culture cells have a marked increase in cytoplasmic ribonucleic acid (RNA) (as seen by acridine orange staining) surrounding the viral DNA inclusions, analogous to the *in vivo* changes observed by Walker (*4*). Although the primary stages of the tissue culture infections do not show the marked hypertrophy, other cytopathic effects—rounding of the cells and increased refractivity—are evident as early as 4 or 5 days after infection. This is in contrast with the slow development of lesions *in vivo.*

Our initial studies, aimed at correlating the morphologic changes with biochemical events associated with *in vitro* replication of this virus, were oriented toward examination of the patterns of macromolecular biosynthesis in the early stages of infection.

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[4] The occurrence of this disease in angelfish has been reported previously by Nigrelli (*14*).

MATERIALS AND METHODS

In Vitro EXPERIMENTS

Tissue cultures.—The host cell line (designated GFS) used throughout these studies was established in 1965 from fin tissue of the blue-striped grunt (*Haemulon sciurus*). Growth was at 25°C in stationary vessels, and the nutrient medium described by Clem *et al.* (*15*) was used.

Virus.—Lymphocystis virus, strain LD, was isolated from tumors of a gray snapper (*Lutjanus griseus*) and propagated by serial subculture in GFS cells. Passage levels of from 52–65 were employed.

Radiochemicals.—^{14}C-L-Leucine-1 (34.1 mc/mmole) and ^{3}H-adenosine (5.2 mc/mmole) were obtained from New England Nuclear Corporation, Boston, Massachusetts. ^{14}C-Adenine-8 (3.7 mc/mmole) was obtained from Volk Isotopes, Chicago, Illinois.

Experimental procedure.—To insure a high synchrony of infection of the tissue culture cells, a high virus-cell ratio was used. In some experiments, monolayer bottle cultures (5×10^{6} cells/culture) were infected with $10^{8.0}$ ID50 of virus, and an adsorption period of 6 hours was allowed. In other instances, the virus was added to dispersed cells, again at a multiplicity of infection of 20, and the virus-cell mixture was distributed into bottles. Again, 6 hours were allowed for adsorption, after which time monolayers had re-formed. After the adsorption period, cultures were washed and fresh growth medium was added.

At 24-hour intervals, 1 μc radioactive material was added to control and infected cultures. Single-label experiments were with ^{14}C-leucine or ^{14}C-adenine; in double-label experiments, the ^{14}C-leucine was used in combination with ^{3}H-adenosine. After incubation with the radioisotope(s) for 1 hour, cultures were washed and the cells dispersed with trypsin and resuspended in saline. A cell count was made with a portion of the cell suspension, and samples were frozen for infectivity assays where applicable. To measured volumes of all suspensions was added cold $HClO_4$ to a final concentration of 0.2 N. The cells were then disrupted by treatment in a Raytheon sonic oscillator, 10 kc, model DF 101, for 1 minute at maximal power. In some cases, the precipitated protein was collected in Millipore filters; in other instances, protein, RNA, and DNA were extracted and separated by the method of Ogur and Rosen (*16*).

Measurement of radioactivity.—The amounts of radioactive materials incorporated were determined with a scintillation counter, model 6801 (Nuclear Chicago Corp., Des Plaines, Ill.), with the use of 12 ml/vial of a mixture of 18 g of 2,5-diphenyloxazole (scintillation grade), 100 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene (scintillation grade), and 116 g of naphthalene dissolved to 1 liter of dioxane spectroquality reagent. Tritium was counted with an efficiency of 15% and ^{14}C with an efficiency of 52%.

Spectrophotometric determinations.—The total amounts of macromolecules were determined by spectrophotometric reactions. The RNA was measured by the orcinol reaction (*17*), the DNA by the Dische-diphenylamine test (*18*), and the protein by the Lowry modification of the folin reaction (*19*).

Sizing and counting.—The cells were counted in some cases in a hemocytometer and in other experiments with a Coulter counter, model 13, with an attached plotter, model J, which gave distribution of particles.

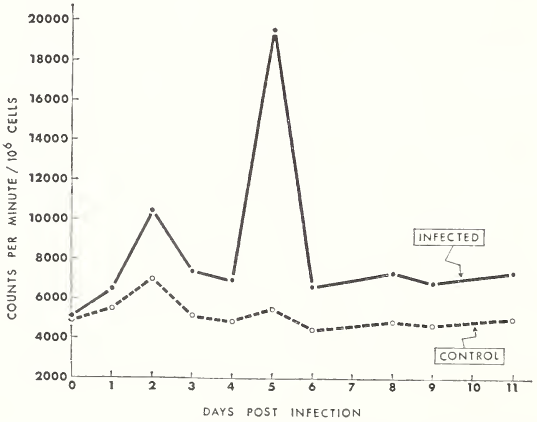
In Vivo Experiments

In the *in vivo* experiments gray snappers affected with lymphocystis disease were used. The tissues were removed from the animals, weighed, and disrupted in 0.05 M phosphate buffer, *p*H 7.5, in a Potter-Elvejhem glass homogenizer, followed by treatment in a Branson sonifier, model S-75, at full power for 3 minutes. Each tissue suspension was separated into 3 portions: One was left untreated, to another was added deoxyribonuclease (Worthington Biochemical Co., Freehold, N.J.) to a final concentration of 40 μg/ml in the presence of 10^{-2} M Mg^{++}, and to the third one was added ribonuclease (Worthington Biochemical Co.) to a final concentration of 20 μg/ml. The tissue suspensions were incubated for 2 hours at 37°C in a water bath, with agitation, and the enzymatic reactions were stopped by addition of 0.2 N $HClO_4$. The Ogur-Rosen procedure of extraction was then followed.

RESULTS

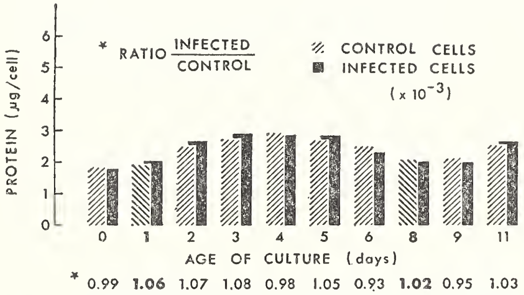
In Vitro Experiments

The profile of the rate of protein synthesis, as depicted in text-figure 1, indicates that the infected cultures had an increased uptake of ^{14}C-leucine with a maximum on the 5th day post infection. The minor peaks that



Text-figure 1.—Profile of rate of protein synthesis in lymphocystis-infected GFS cells. This is a representative experiment of 6 similar assays in which the cultures were harvested on consecutive days after 1-hour pulse labels with ^{14}C-L-leucine. Extraction was performed in some cases with precipitation with 0.2 N $HClO_4$ and collection in Millipore filters, and in others with the Ogur-Rosen procedure; similar results were obtained with both procedures.

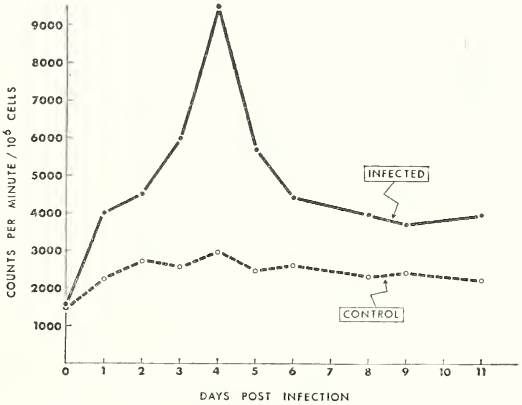
appeared on the 2d day in both control and infected cultures are attributed to the feeding procedure. When the total amounts of protein in control and lymphocystis-infected GFS cells were studied, it was surprising that in the very early stages of infection there was no accumulation of excessive amounts of protein with respect to control cultures (text-fig. 2). This phenomenon was consistently observed.



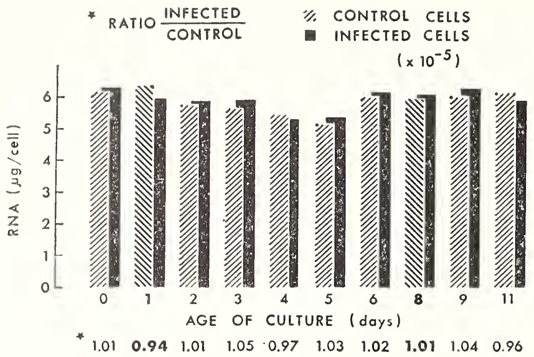
TEXT-FIGURE 2.—Total amounts of protein in control and lymphocystis-infected GFS cells, as determined by the Lowry modification of the folin reaction.

The rate of synthesis of RNA (text-fig. 3) indicated that there was a significant increase in the incorporation of the radioactive precursor in the infected cells with a peak on day 4, one day before the observed maximum synthesis of protein. The total amounts of RNA (text-fig. 4) showed no increase over those of uninfected control cells.

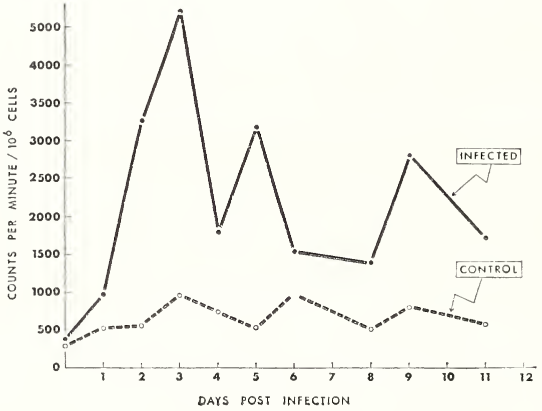
The profile of DNA synthesis is shown in text-figure 5. Three peaks are evident in the infected cultures. Although we cannot explain the significance of these peaks, this type of profile was obtained consistently. When the results of the pulse-label experiments were expressed as cumulative values (text-fig. 6), they showed a straight line of a significantly increased



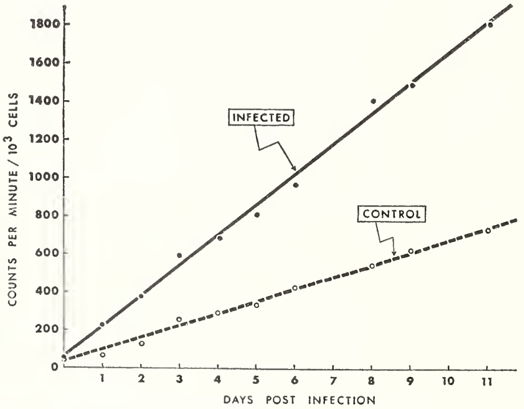
TEXT-FIGURE 3.—Profile of rate of RNA synthesis in lymphocystis-infected GFS cells. The cultures were labeled for 1 hour with ^{3}H-adenosine and the RNA was separated by the Ogur-Rosen procedure.



TEXT-FIGURE 4.—Total amounts of RNA in control and lymphocystis-infected GFS cells, as determined by the Mejbaum adaptation of the orcinol reaction.



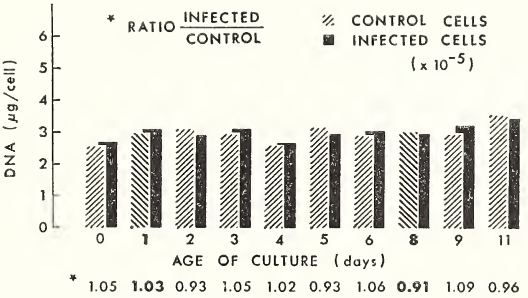
TEXT-FIGURE 5.—Profile of rate of DNA synthesis in a representative experiment with lymphocystis-infected GFS cells. The uptake of radioactive precursor was measured with a scintillation counter after the extraction of the DNA by the Ogur-Rosen method.



TEXT-FIGURE 6.—Cumulative results of the incorporation of ^{14}C-adenine in DNA in lymphocystis-infected GFS cells in comparison with control cultures.

rate of synthesis in infected cultures as compared with controls. When the total amounts of DNA in infected and control cells were measured, once again there was no accumulation of the newly synthesized macromolecules despite the heightened rate of synthesis (text-fig. 7). Identical results were obtained in single-label experiments employing ^{14}C-adenine and in double-label experiments where tritiated adenosine was used as one of the labels.

Measurements of cell size in the Coulter counter revealed no significant differences in the infected and control culture populations. The biochemical findings are thus compatible with the morphologic observations during the early phases of infection.



TEXT-FIGURE 7.—Total amount of DNA in control and lymphocystis-infected GFS cells as estimated by the Dische-diphenylamine reaction.

In Vivo Experiments

Although the lymphocystis tumor has been the subject of numerous histologic examinations, very little is known about its chemical composition. Inasmuch as the tumor consists morphologically of grossly enlarged cells, it would follow that the cells should contain commensurately increased amounts of constituents. To test this relationship, we subjected tumor tissue to chemical analysis and compared it with normal skin. In these experiments, each of the nucleic acids was subjected to the action of nucleases to ascertain that our fractionation procedure, with the Ogur-Rosen method, yielded the desired separation of the macromolecules.

The biochemical assays (table 1) indicate that the tumors contained substantially greater amounts of RNA and DNA per gram of tissue than did normal skin, but only a slightly increased amount of protein. The results presented in the table represent an average of 4 experiments, each of which showed the same differences between tumor and normal tissue as regards their content of the macromolecules measured. It is to be pointed out that the results of the assays are expressed as values per gram wet weight of tissue rather than on a cell population basis. Because the tumors consist of grossly hypertrophied cells, one is justified in assuming that the observed differences are minimal values.

The results also attest to the integrity of the preparations, since treatment with ribonuclease diminished the activity of the RNA preparation

TABLE 1.—Total amounts of macromolecules in fish skin and lymphocystis tumor *in vivo*

Macromolecules	Tissue	μg/g wet weight of tissue		
		Untreated	Ribonuclease-treated	Deoxyribonuclease-treated
Protein	Tumor	7.5 × 10³ μg	7.6 × 10³ μg	7.7 × 10³ μg
	Skin	6.4 × 10³ μg	6.1 × 10³ μg	6.2 × 10³ μg
RNA	Tumor	846 μg	167 μg	832 μg
	Skin	463 μg	125 μg	472 μg
DNA	Tumor	709 μg	696 μg	265 μg
	Skin	244 μg	244 μg	109 μg

but had no effect on the DNA fraction. Conversely, treatment with deoxyribonuclease had an equally specific effect. The two nucleases caused no changes in the protein fraction.

DISCUSSION

The lymphocystis virus has many interesting features. First it is one of the few viruses known to be associated with disease of fishes that has been isolated and characterized in tissue culture. Although the virus has a DNA nucleoid, it replicates in the cytoplasm of the cell, a feature shared by two other groups of viruses native to cold-blooded animals—the frog viruses and the iridescent group of insect viruses. This interesting and important characteristic is not limited to viruses of poikilothermic animals, however, inasmuch as members of the large family of mammalian and avian DNA-containing poxviruses have a similar site of replication.

Recent studies have disclosed several interesting sequences in the replication of the poxviruses. According to Kates and McAuslan (*20*), these viruses contain an RNA polymerase which presumably initiates the production of viral messenger RNA. Information of this nature is still lacking for the cytoplasmic DNA viruses of cold-blooded animals. Studies such as the ones reported in the present paper should help elucidate the replicative events of the lymphocystis virus.

Perhaps the most interesting aspect of infection with lymphocystis virus is its effect on the cell. The infected cells are transformed in that they undergo an enormous hypertrophic alteration. In addition, they acquire a hyaline capsule and accumulate large amounts of DNA and RNA. Such cells cease to divide, and the tumors which develop *in vivo* are the result of cellular hypertrophy rather than cellular proliferation. In our experience, tumors have been found sporadically in marine fishes and have also occurred in epidemic form. The conditions predisposing to tumorigenesis are not known.

Lymphocystis virus has been studied in tissue cultures of cells derived from several species of fish. In our laboratory, infections have been produced in cell lines of snapper fin and of grunt kidney and fin. (Natural disease in grunts was not observed.) We have noted a difference in the susceptibility to the virus of different cell lines; one of our grunt fin lines is totally resistant.

The present results are based on studies in a highly susceptible line of grunt fin cells. Under certain conditions, especially with a low multiplicity of infection and prolonged observation, the virus has brought about cellular hypertrophy analogous to that seen in the fish tumors. In the present experiments, in which a high multiplicity of infection was used, such hypertrophy was not seen during the 11-day observation. All cells showed signs of infection, however, in that they were rounded and became refractile.

The kinetic data revealed significant increases in the rates of synthesis of protein, RNA, and DNA, with a peak of RNA synthesis preceding the maximum production of protein—yet there was no accumulation of these macromolecules in the cells. It thus appears that the increased rates of synthesis were probably associated with increased rates of degradation. As previously mentioned, cellular giantism has been observed in other experiments in which low multiplicities of infection were used. Although biochemical assays of cells were not made in these experiments, staining with acridine orange revealed accumulation of cytoplasmic RNA.

The reasons for the difference in response to varying virus inputs are unknown. We are currently collecting cellular materials at various stages of infection to characterize the DNA and RNA of the infected small cells and of the giant cells. We are also pursuing additional work on the morphologic changes that result from high and low multiplicities of infection. There is an obvious need for additional electron microscope examinations of cells in relation to both multiplicity of infection and time. Such studies should assess the physiologic modifications of the cell as well as measure the extent of virus replication and accumulation.

Cultures infected with low multiplicities differ from those reported here in still another respect. Whereas in the present experiments all cells showed cytopathic effects of infection, with a low multiplicity of infection one often sees a relatively small number of giant cells in a monolayer of perfectly normal cells (*see* fig. 4). The question therefore arises: Does infection result in the production of interferon which protects the remaining cells? This question is currently under study.

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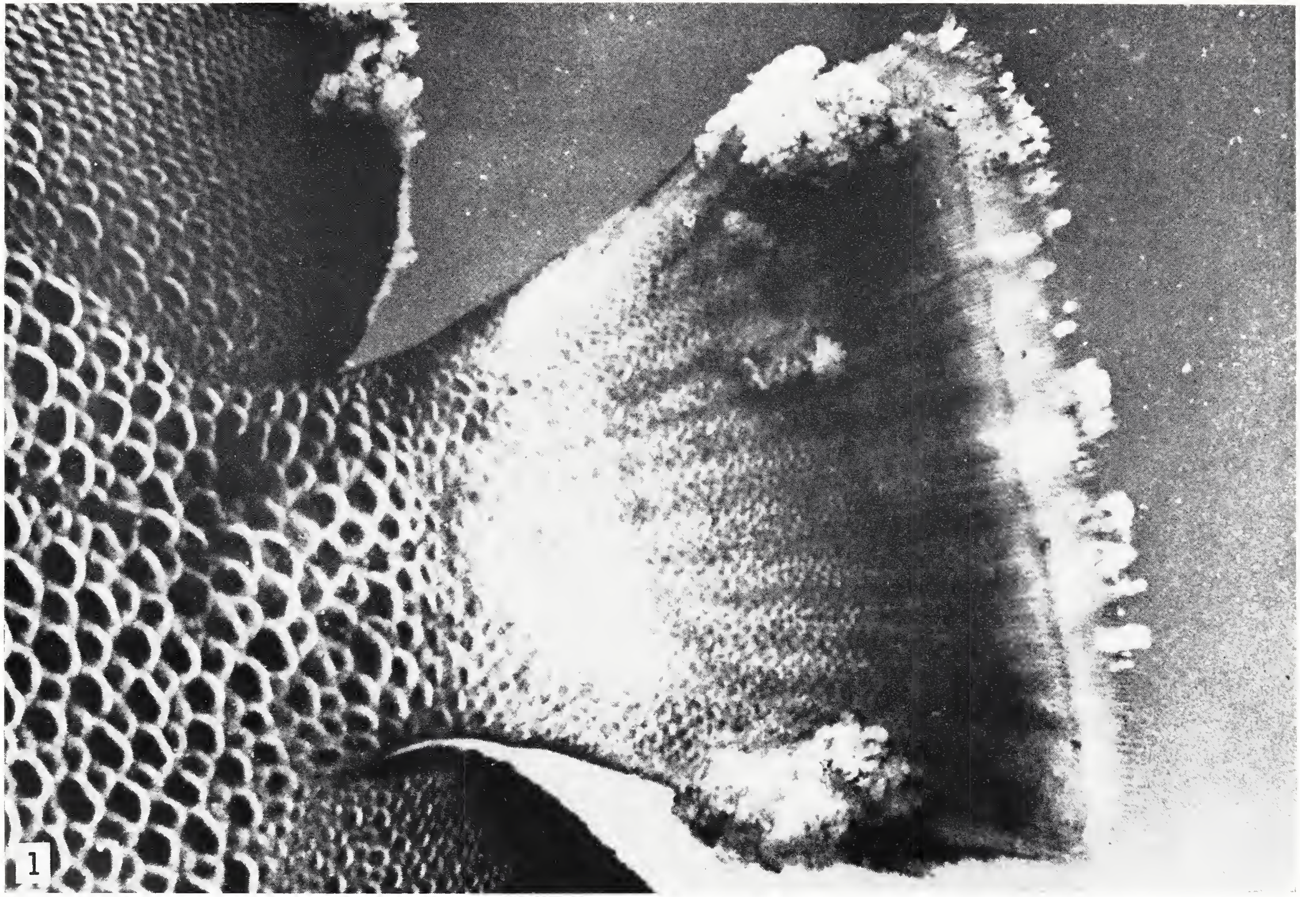

FIGURE 1.—Gray angelfish (*Pomacanthus aureus*), captured in the Florida Straits, with lymphocystis disease manifested in tumors on the tail and fin.

Courtesy of The Miami Seaaquarium, Inc.

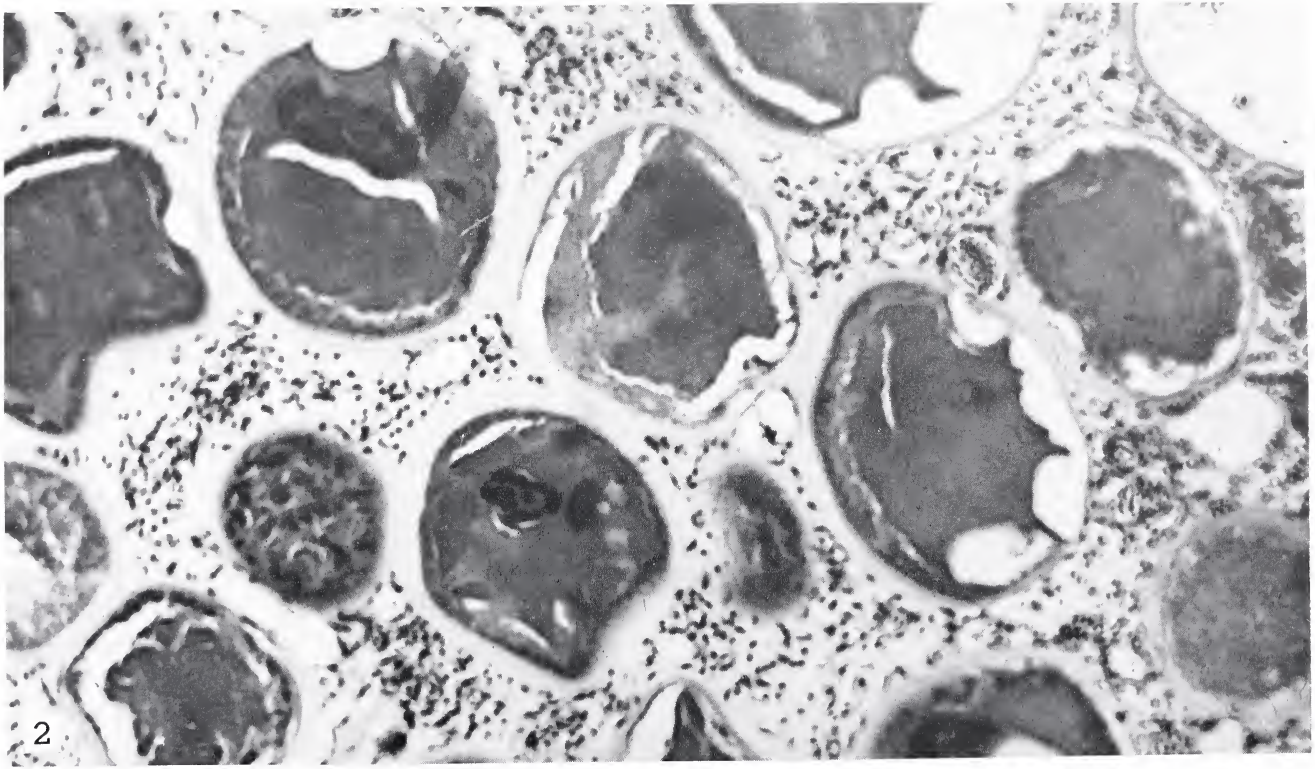

FIGURE 2.—Histopathology of lymphocystis tumor. Viral cytoplasmic inclusions are evident as is capsular structure characteristic of advanced stages of infection. Comparative sizes of normal and infected cells are clearly evident. Hematoxylin and eosin.

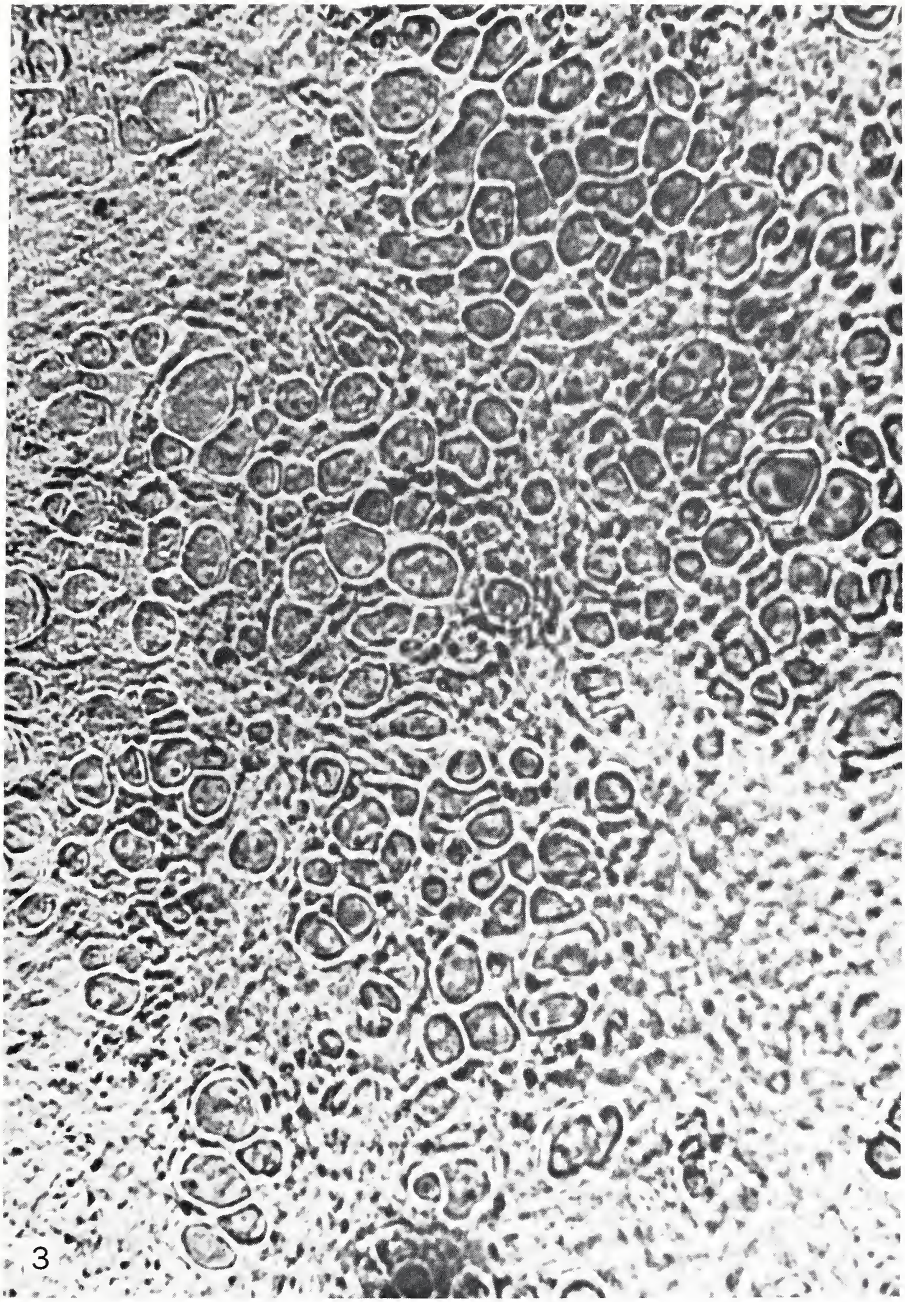

Figure 3.—Low-power view of unstained lymphocystis-infected tissue culture in which most cells show cytopathic effects.

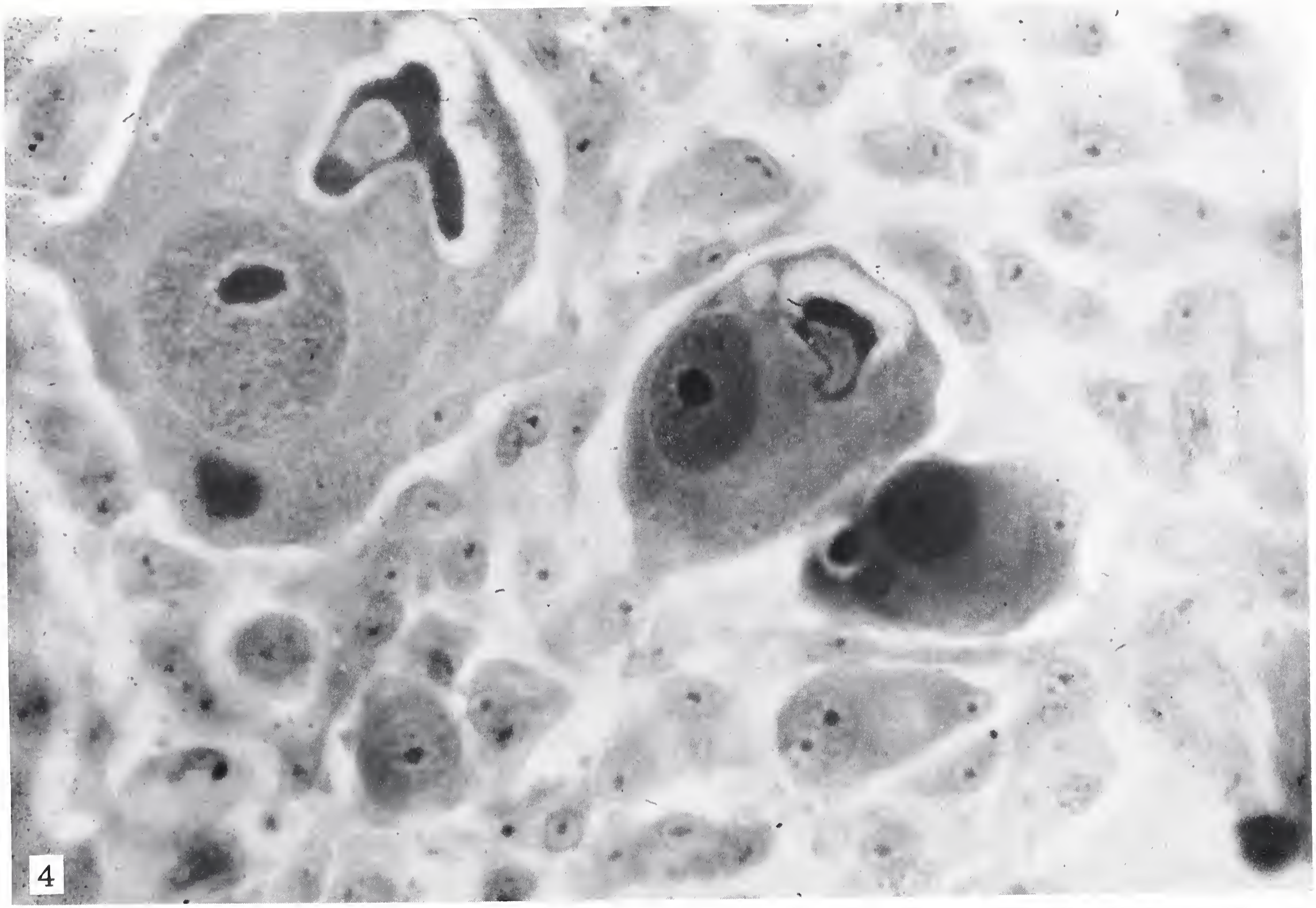

FIGURE 4.—Advanced stage of infection of marine fish tissue-culture cells with lymphocystis virus (low multiplicity of infection). *Note* progressive increase in cell size from normal surrounding cells to the giant cell (*upper left*). Cytoplasmic inclusions are clearly visible, especially in the 2 largest cells. Hematoxylin and eosin.

Microsporida and Tumors, With Particular Reference to the Lesion Associated With *Ichthyosporidium* Sp. Schwartz, 1963[1]

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SUMMARY

Most microsporidian infections are accompanied by local swellings, often called "tumors." The swellings are due to multiplication of parasites and to host cell response. Usually, there is hypertrophy of the infected cells, involving either nucleus or cytoplasm, or both. The best known of the many manifestations of cell hypertrophy is the *Glugea* "cyst," a single infected cell which reaches a diameter of 4 mm and which, along with certain other "cell hypertrophy tumors," Weissenberg has called a "xenoma." The xenoma is a symbiotic complex. Other Microsporida elicit various types of response from noninfected host cells. A case involving unusual and atypical mitosis in noninfected cells of the fat body of *Blatta orientalis* harboring *Plistophora blattae* was said to represent neoplasia of the adipose tissue, although it is doubtful that many modern authors would use "neoplasia" in that case. Another type of response by noninfected host cells accompanies infections of fish with Microsporida which are of uncertain generic affinities but are often placed in genus *Ichthyosporidium*. A case report on the large "tumor" in *Leiostomus xanthurus* accompanying infection with *I.* sp. Schwartz, 1963, is presented. The "tumor" is a large, multicystic lesion due to massive microsporidian infection in the deep tissue. There is a correspondingly massive host response, including infiltration of infected tissue by numerous migratory cells and formation of multinucleate giant cells. Simultaneous growth of the numerous cysts (due to proliferation of the parasites within each) and increase in number of host cells (due to infiltration and, perhaps, proliferation of migratory cells) account for the great size of the "tumor." Late developmental changes include vascularization of the altered host tissue and, frequently, rupture of some cysts with subsequent phagocytosis of the escaping parasites.—Nat Cancer Inst Monogr 31: 237–249, 1969.

SPECIAL INTEREST in the relationship of Microsporida to tumors has developed during the present decade after the occurrence of Microsporida in mammals was demonstrated. Morris *et al.* (*1*) found ascites and hepatosplenomegaly in mice associated with protozoan-like cytoplasmic structures. Nelson (*2*) and Lainson *et al.* (*3*) demonstrated that the protozoan-like bodies are microsporidan spores and suggested their possible identity with *Encephalitozoon cuniculi* Levaditi, Nicolau and Schoen, 1923, in different mammals. Lainson *et al.* (*3*) and Weiser (*4*) independently in the same year placed this microsporidan in genus *Nosema*, suppressing the name *Encephalitozoon* as a junior synonym. While Nelson found that a *Nosema*, perhaps *N. cuniculi*, causes ascites in mice, Petri (*5, 6*) and Petri and Schiødt (*7*) found that a *Nosema* grows in the cells of Yoshida rat sarcoma (a transplantable, malignant ascites tumor), inhibits the cancer growth, and confers a degree of immunity on the rat host. Furthermore, Arison *et al.* (*8*) found that the microsporidan which produces murine ascites has an inhibiting effect on a variety of mouse tumors. Although it is relevant to note that Microsporida seem clearly to have special relationships to some malignancies in mammals, further consideration of this subject is beyond the scope of a symposium on tumors of lower animals.

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[1] Contribution No. 364, Natural Resources Institute of the University of Maryland.

The subject of lesions associated with Microsporida has not been treated in a comprehensive manner, perhaps the most complete treatment being a compilation of data presented by Kudo (*9*). Furthermore, a great many of the lesions have not been studied critically. Many workers, not being pathologists, have concentrated their attention on describing a few stages in the life cycle of the parasite, especially the sporogony stages. While these workers often observed physical changes in the infected host, their observations were usually rather casual. Nevertheless, a large amount of information is available but scattered in the literature. While a complete summary of these data might be pertinent to the present study, the subject is too large for adequate treatment at this time. The scope of the present paper must be limited to reviewing briefly some of the most pertinent or most thorough studies on Microsporida in relation to tumor-like lesions, and presenting a case report.

While some microsporidan infections, such as *Nosema* sp. Sprague, 1965 (*10*), in the blue crab, become generalized, a great many others seem to remain localized. With rare exceptions, an example being *Octosporea effeminans* Bulnheim and Vávra, 1968 (*11*), a local infection is accompanied by a lesion of some sort. Perhaps the simplest lesions are certain ones associated with microsporidan infections of the gut epithelium of insects. Examples of the latter are those caused by *Plistophora kudoi* Sprague and Ramsey, 1942 (*12*), and *Nosema termitis* Kudo, 1943 (*13*). These parasites inhabit the epithelial cells where they reproduce in large numbers and cause mechanical distention of the cytoplasm. Blobs of the cytoplasm of infected cells, extending into the lumen and containing parasites, break off. The nuclei of the host cells remain unchanged and no other pathological accompaniments are evident. Such microsporidan lesions are rare, however, and appear to have little relevance to the study of tumors.

Most microsporidan infections are accompanied by swellings at the sites of infection. The swellings have frequently been called "tumors." In this connection the word has generally been used in a broad sense, as defined by

Steinhaus and Martignoni (*14*), to apply to a swelling without particular reference to its fundamental nature. Since many pathologists, like Warren (*15*), seem to consider "tumor" as essentially synonymous with "neoplasm" ("an uncontrolled new growth of tissue"), it is pertinent to consider the nature of microsporidan "tumors" and try to discover what relevance, if any, they have to the study of tumors *sensu stricto.*

Two main components contribute to the size of the swelling: the increasing number of parasites and the response of the host cell. The response usually involves increase in size of infected cells. These, as Kudo (*16*) recently stated, "show usually an enormous hypertrophy of both cytoplasm and nucleus . . ., a characteristic feature of the microsporidan infection." In an earlier work (*9*) the same author said: "One of the most conspicuous changes an infected cell undergoes is the striking hypertrophy of its nucleus which may become enormously enlarged, may increase in number or may show various ways of division." Another kind of host response is infiltration of cells into the infected tissue and/or proliferation of cells. It is this second type of response which is illustrated by the case report presented in this paper.

Undoubtedly, the most intensive studies of microsporidan "tumors" have been made by Weissenberg, who, during a half century of research, has given primary attention to the "cell hypertrophy tumors" or "xenomas" and published numerous papers on them (*17–27*). While some of his major works have dealt with the hypertrophied cells of lymphocystis disease, he has done classic studies also on those associated with certain Microsporida of fish. *Nosema lophii* (Doflein, 1898) Pace, 1908, occurs in the ganglion cells of the central nervous system of *Lophius piscatoris* and *L. budegassa.* It produces swellings which are composed of numerous xenomas and resemble bunches of grapes. The nuclei of these xenomas, although greatly hypertrophied and lobed, remain undivided. *Glugea anomala* (Moniez, 1887) Gurley, 1893, produces conspicuous (3–4 mm) *Glugea* cysts, which arise from migratory mesenchyme cells, in *Gasterosteus aculeatus.* According to Weissenberg (*27*), "the amitotic development of numerous nuclei as descendants from the single hypertrophied nucleus of the host cell represents a characteristic peculiarity of the *Glugea* xenomas in the cytopathology of fish diseases." Weissenberg (*22–24*, *27*) conceives of the xenoma as a symbiotic complex, consisting of host cell component and parasite. He believes that the nucleus of the host cell is transformed rather than degenerated.

Glugea weissenbergi Sprague and Vernick, 1968 (*28*), in the fish *Apeltes quadracus* is very similar to *G. anomala* and the authors agree with Weissenberg about the nature of the xenoma. After a light and electron microscope study, however, they postulated that the earliest stages of the parasite are intranuclear. Membranes of the nuclei of the host cell seem to have branches extending into the cytoplasm which resemble annulate lamellae and these seem to be continuous with the endoplasmic reticulum. The *Glugea* apparently develops within this nucleo-cytoplasmic continuum, the nuclear end of the continuum being a factory which reduplicates the genome of the *Glugea*, while the annulate lamellae furnish the membranes characteristic of the young parasite. The young parasites then complete their development to the spore stage in the cytoplasm and cytoplasmic vacuoles of the host-cell component. The authors believe *Glugea* has some similarities to some viruses and raise the question of a possible interaction between the genomes of the host cell and parasite.

The cytopathological changes accompanying most microsporidan infections may be essentially limited to infected cells. As already pointed out, however, some species elicit a greater host response. One of these is *Plistophora blattae* Weiser, 1960, in the adipose tissue of the cockroach, *Blatta orientalis*. Mercier (*29*) found the parasite to be present in a state of diffuse infiltration, giving the tissue a white, instead of the normal transparent, appearance. The abdomens of the insects were distended. While mitosis was never seen in adipose cells of normal adult roaches, some adipose cells of infected specimens underwent abnormal, asymmetrical, multipolar, or irregular mitosis. Although Kudo (*9*), referring to Mercier's work, said, "the nucleus of the infected host cell undergoes abnormal mitotic division," I understand that Mercier observed the mitosis in noninfected cells. Mercier referred to the proliferation of cells as neoplasia of the adipose tissue ("Néoplasie du tissu adipeux").

The present case report concerns a large swelling associated with *Ichthyosporidium* sp. Schwartz, 1963. This parasite is very similar to *Pleistophora gigantea* (Thélohan, 1895) Swellengrebel, 1911, also known as *Ichthyosporidium giganteum* (Thélohan, 1895) Swarczewsky, 1914, which produces a large abdominal "tumor" in fish of genus *Crenilabrus*. Although some authors, notably Kudo (*16*), have followed Swarczewsky (*30*) in considering the latter species a haplosporidan, Sprague and Vernick (*31*) recently confirmed the view of Thélohan (*32*) and Swellengrebel (*33–35*) that it is a microsporidan. Schwartz (*36*) also regarded the parasite he discovered as a haplosporidan, but Sprague (*37*) examined his material and found it to be a microsporidan. More material of the same kind has become available, and a series of studies on the parasite and its relation to the host is underway at this laboratory. Specimens have been submitted to the Registry of Tumors of Lower Animals, Smithsonian Institution, and have been assigned the accession number RTLA 103.

MATERIAL AND METHODS

Infected host fish, *Leiostomus xanthurus*, were collected in Chesapeake Bay, Maryland, by Douglas E. Ritchie of Chesapeake Biological Laboratory. One was brought back to the laboratory in formalin and others were brought under refrigeration. Material from the latter was studied in the fresh condition to note the characteristics of the spores and to demonstrate the everted polar filaments. Portions of the tumors were then fixed. Smears and sections, fixed and stained by a variety of methods, were prepared for future study. Observations mentioned in this report were based largely on sections which were fixed in 10% formalin, alcoholic Bouin, Zenker, or formalin-alcohol-acetic mixture and stained with Heidenhain's iron hematoxylin.

OBSERVATIONS

This parasite produces 1–3 large (2–5 cm long) ovoid masses, oriented in an anterio-posterior direction, in the ventral abdominal wall of the host fish *Leiostomus xanthurus* (fig. 1). Since the masses in the few fish examined were located between the skin and the abdominal muscles and were sometimes surrounded by adipose cells (fig. 2), the subcutaneous connective tissue is probably the usual site of infection. Presumably, the young parasite is intracellular, but organelles indicative of the presence of an infected host cell could not be recognized. Further study is needed to identify the host cell and determine its fate. In one fish with a large swelling, a few muscle cells (fig. 3) were parasitized. These parasites, however, may belong to a different species because their nuclei, having several small peripheral granules instead of the usual single, large granule in a nucleus not clearly vesicular, are atypical.

Most, if not all, developmental stages of the parasite may occur in the same lesion. When a section of a lesion contains several stages, these occur as large patches differing in appearance according to the stage of development of the parasites, while each patch contains parasites that are roughly in the same stage. The earliest recognized stages (fig. 2 at *lower right* and fig. 4) are small, irregular, multinucleate bodies about 15–20 μ in diameter. These tend to occur in groups of 2 or more and typically have paired nuclei. Their limiting membranes are relatively thin and appear to be continuous with fibrous elements of the connective tissue. Around these parasites is a particularly dense accumulation of migratory cells.

Later developmental stages of the parasite become progressively larger and are sharply defined by distinct fibrous capsules (fig. 5). These may appropriately be called "cysts." The cysts vary in appearance but frequently are subdivided, with the subdivisions often developing asynchronously. Typically, one or more of the subdivisions contain a large vacuole.

The cysts continue to grow and become enormously distended with sporogony stages (figs. 6 and 7). While the interior of the cyst contains mostly mature spores, sporoblasts and some other pre-spore stages typically are present around the periphery (fig. 7). The spores are typical microsporidan spores, a conclusion which I previously (*37*) reached after observing periodic acid-Schiff (PAS) preparations of Schwartz and confirmed in the present study by observing the everted polar filament of fresh spores.

As the cysts become mature and very large, they may cease to appear as discrete entities in sections of the lesion. Instead, the lesion may appear to consist of a large mass of spores penetrated by trabeculae (fig. 8). These trabeculae are limited by the capsules of the cysts and contain the consolidated tissue which presumably develops from the subcutaneous connective tissue and immigrating cells. As the mass enlarges, some of the capsules may rupture and leave large masses of the parasite without limiting membranes (fig. 9).

Especially noteworthy are the developmental changes in the infected tissues. As already mentioned, infection of the connective tissue by the young parasite is soon followed by a massive invasion of migratory cells into the parasitized tissue. These cells apparently proliferate and eventually produce a considerable amount of new tissue between the cysts. During the proliferation, karyokinesis is rapid. This appears to be amitosis, although it may be atypical mitosis. Cytokinesis does not always follow immediately after karyokinesis, and this results in giant cells with many small nuclei (fig. 10). In spite of diligent search, no evidence that these cells are parasitized could be seen. As the parasite matures and sporulates, the new tissue developing between the cysts also takes on a different character. Rapid cellular proliferation ceases and the cells become mostly uninucleate, with nuclei approximately uniform in size. At the same time, the new tissue often becomes quite vascular.

It now appears that the lesion has two main components, the masses of parasites and the altered host tissue, both of which increase in bulk and contribute to the ultimate great size of the swelling.

Sometimes the new tissue is infiltrated with the parasite in various stages of sporogony. These infiltrating parasites, which presumably have escaped through the ruptured capsule, are often intracellular, as Sprague (*37*) previously observed. This may be the result of phagocytosis.

DISCUSSION

There may be a fundamental difference between the many species of Microsporida which stimulate hypertrophy, often followed by fragmentation, of the nucleus of the infected host cell and the few which do not. Kudo (*38*) remarked: "The cause of the hyperthrophy of the host nuclei is obviously the presence of the microsporidan, and the process appears to be a manifestation of increased metabolic activities on the part of the infected host cell as a reaction to the stimulus exercised by the parasite. But the exact mechanism of the hypertrophy remains unknown." As already pointed out, Sprague and Vernick (*28*) suspect that early stages of *Glugea weissenbergi* are intranuclear. They postulate that the hypertrophied nucleus is a factory in which the genome of the *Glugea* is reduplicated and raise the question of a possible interaction of the DNA with that of the parasite. If the occurrence of intranuclear development of stages of some Microsporida should be confirmed, it might account for the difference between species which stimulate hypertrophy of the nucleus of the infected cell and those which do not. At the same time, it might be a step toward understanding the mechanism of the hypertrophy.

Schuberg (*39*), referring to the cells of the testis of a fish "Barbe" infected with *Plistophora longifilis*, said that the hypertrophied nuclei reminded him of the giant nuclei of many sarcomas. This suggests the possibility that the mechanisms of nuclear hypertrophy in cells infected with Microsporida might have something in common with that of the giant nuclei of the sarcomas. Therefore, further information on the relation of Microsporida to hypertrophy of nuclei of infected cells might be of interest to oncologists.

Convincing evidence that Miscrosporida produce true neoplasms, in the sense of transplantable tumors, is lacking. Mercier (*29*) spoke of neoplasia of the adipose tissue of a cockroach infected with *Plistophora blattae*. It is most logical to conclude that the new growth was merely a host response to the presence of the parasite, not an uncontrolled transplantable growth. Perhaps the lesion accompanying infection with *Plistophora blattae* merits further study.

The lesion accompanying infection with *Ichthyosporidium* sp. is a large multicystic lesion due to massive miscrosporidan infection of the deep tissues. The host response is correspondingly massive. It has been noted that the host of *Ichthyosporidium* sp. responds in several ways to the invading parasite. There is encapsulation of the young parasite accompanied by cellular infiltration of the parasitized tissue. There are changes in the infected tissue, involving proliferation of the invading cells with giant cell formation and increased vascularization. Thus, each mass of parasites in an original focus becomes surrounded both by a capsule and by the infiltrating and proliferating cells. Finally, there is phagocytosis of those parasites which escape into the surrounding tissue if the retaining capsule ruptures. This response of the host tissue is an orderly process of change appropriate to the presence of the developing parasite. There is no evidence of an "uncontrolled new growth of tissue" in the sense of a tissue which might continue to grow in the absence of the parasite. It appears that the host's response to the invading parasites by building a wall around them is not detrimental to the invaders. On the contrary, it protects them from being phagocytized, while the increased blood supply to host tissues probably serves not only certain needs of the host but also the needs of the parasites.

A distinction must be made between the type of cell hypertrophy represented by the *Glugea anomala* xenoma (an infected cell) and that seen in some of the migratory cells, apparently noninfected, which infiltrate the tissues invaded by *Ichthyosporidium* sp. As Weissenberg (*21*) demonstrated, the former involves a symbiotic association of parasite and host cell which grows to great size. The latter type of hypertrophy, involving apparently noninfected cells, appears to be similar to the giant cell formation that frequently accompanies the presence of parasites and other foreign bodies. The two mechanisms of hypertrophy may be quite different.

Although both processes may result in multinucleate cells, one is the transformation of a single cell while the other is said to be in some instances a fusion of cells. Mercier (*40*), studying *Glugea gigantea* Thélohan, most often called *Ichthyosporidium giganteum* (Thélohan), made a distinction similar to that which I have just made between the two types of cell hypertrophy. Mercier said: "Les cellules géantes des tumeurs à *G. gigantea* ont donc une signification toute différente de celle des cellules géantes qui entrent dans la constitution des plasmodes ou des kystes à noyaux géants des soi-disant *Myxocystis*, de *Glugea anomala*, etc. Dans les premiers cas la cellule géante correspond uniquement à une réaction de défense de l'organisme, tandis que dans le second elle constitue un complexe symbiotique avec le parasite."

Ichtyosporidium sp. Schwartz is almost identical in its morphology, as well as in host-parasite relations, with the species which Swellengrebel (*33–35*), Swarczewsky (*30*), and Mercier (*40*) studied. The two species, having spores of slightly different size and having different hosts, are probably distinct but are unquestionably congeneric. To what genus they belong remains an open question. Sprague (*37*, *41*) and Sprague and Vernick (*31*) have already solved some of the taxonomic problems involving these unusual parasites. It is hoped that studies now in progress will reveal details of the life cycle needed to judge whether species of *Ichthyosporidum*, a microsporidan genus, have distinctive characters which justify retention of this taxon.

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All figures represent tumors associated with *Ichthyosporidium* sp. Schwartz in the fish *Leiostomus xanthurus*. All except figure 1 are from sections.

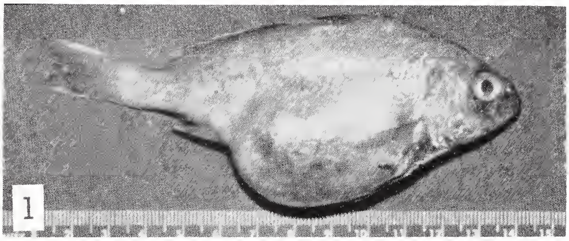
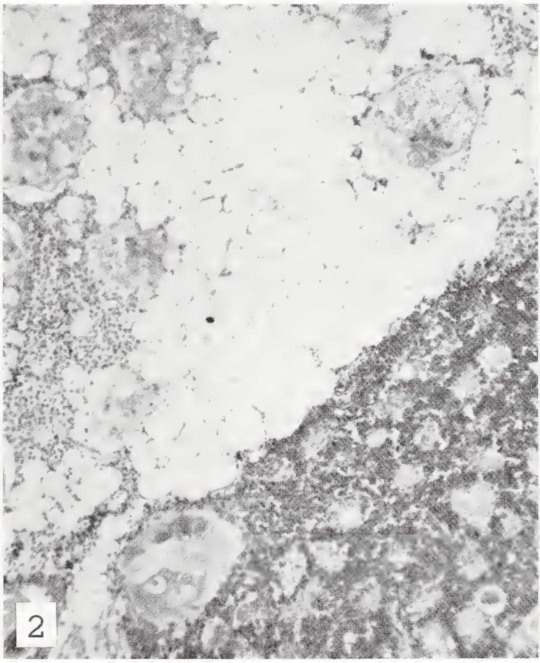


FIGURE 1.—*Leiostomus xanthurus* with large abdominal tumor.



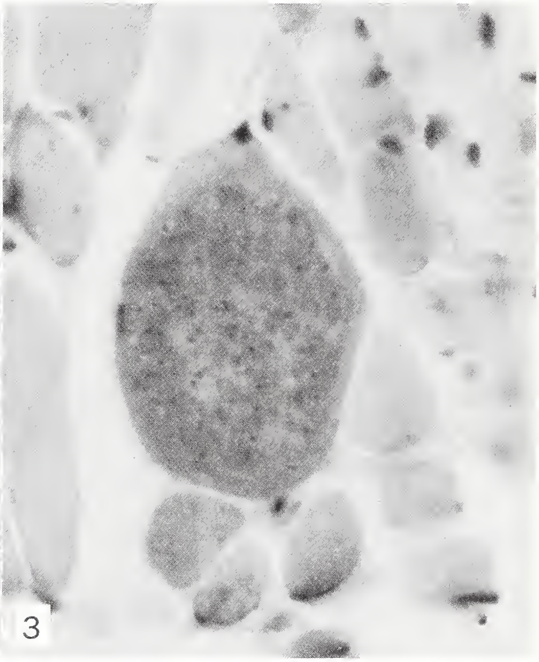
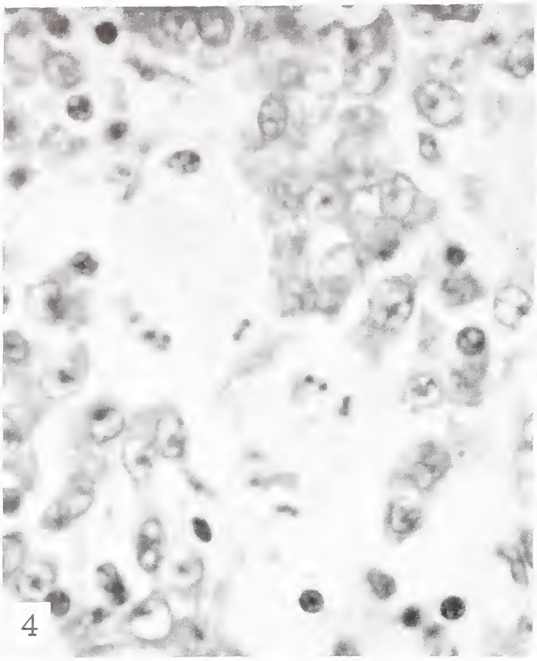
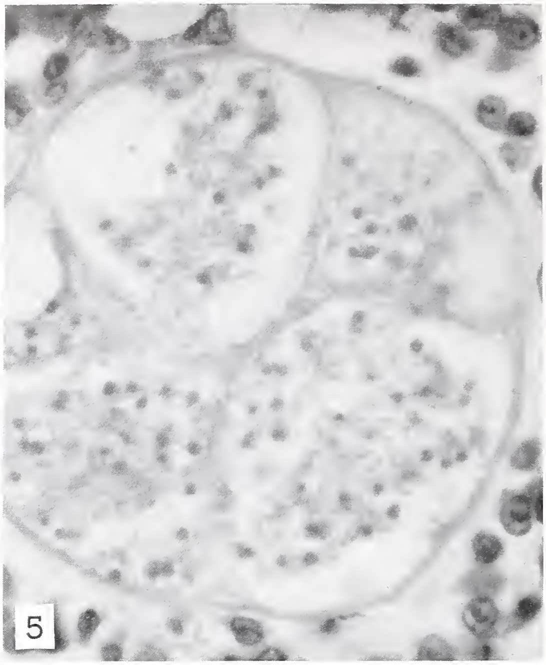
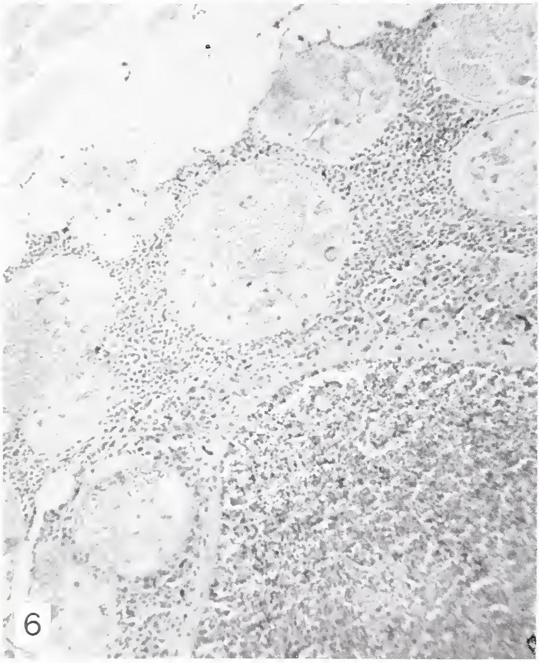


FIGURE 2.—Section through portion of tumor in early stages of development in subcutaneous connective tissue. At *lower right* is heavy concentration of parasites in very early stages. *Above* are a few slightly older parasites. All parasites are surrounded with a dense concentration of infiltrating cells. Formalin-Heidenhain. × 130

FIGURE 3.—A rare instance of an infected muscle cell associated with a tumor. Whether the parasite is *Ichthyosporidium* sp. is doubtful. Formalin-alcohol-acetic mixture Heidenhain. × 1300







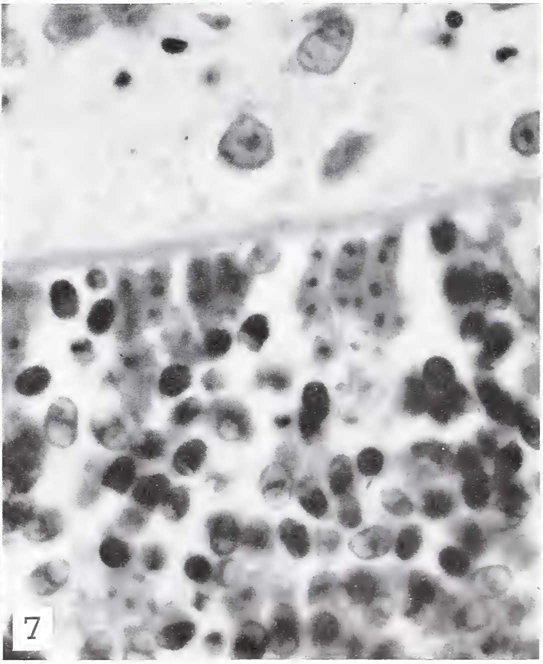
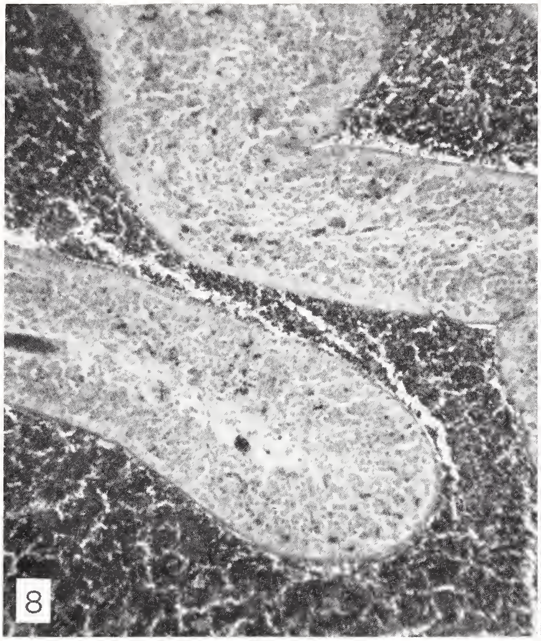


FIGURE 4.—Enlargement of portion of tumor seen at *lower right* of figure 2. Two very young multinucleate parasites, with paired nuclei, appear in *center*. Encapsulation of parasites by fibrous elements of connective tissue has barely begun. × 1300

FIGURE 5.—More advanced early stage, now encysted. A distinct fibrous capsule has been formed. Cyst has several components, probably representing several originally infected cells encysted in a common capsule. A vacuole is usually present in each encysted individual. Formalin-Heidenhain. × 1300

FIGURE 6.—Portion of a mature cyst is shown at *lower right* and immature ones appear *above*. Tissues seen between cysts develop into "trabeculae" referred to elsewhere. Formalin-Heidenhain. × 130

FIGURE 7.—Enlargement of portion of margin of mature cyst seen in figure 6. Stages in sporogony, including many mature spores, enclosed by capsule. × 1300



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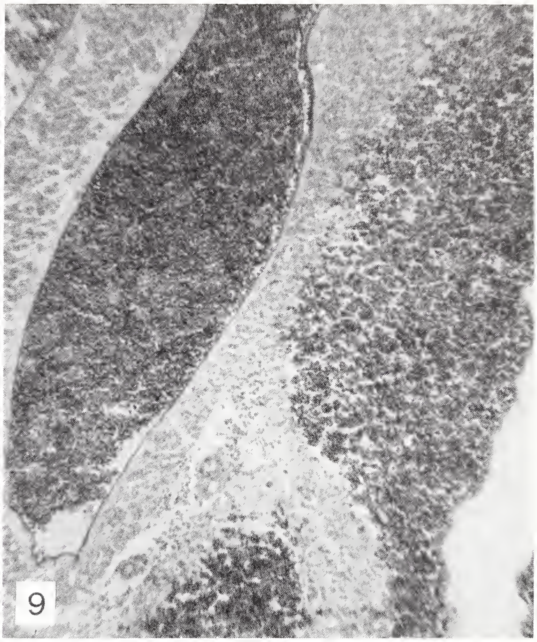

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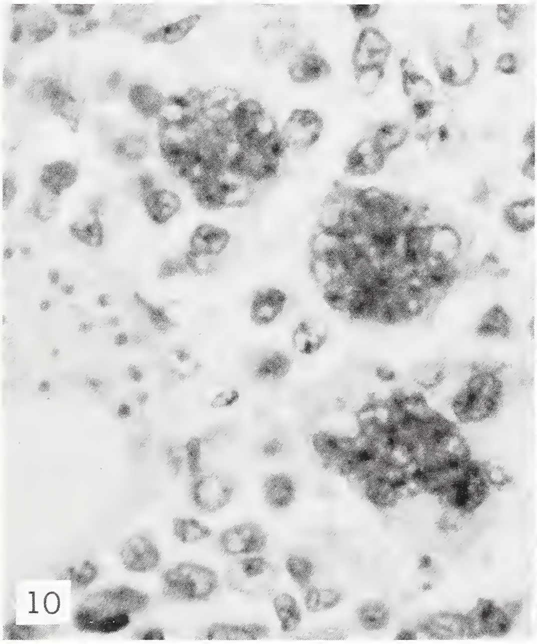

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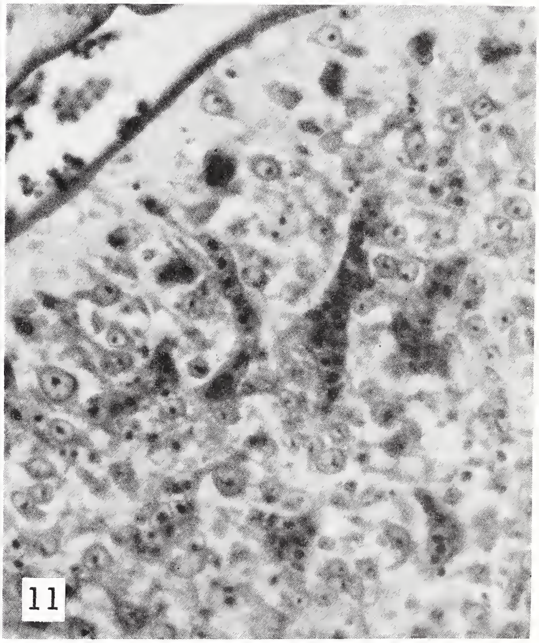

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FIGURE 8.—Trabeculae in large, mature tumor. These consist of host tissue bounded by capsules of distorted, mature cysts. Zenker-Heidenhain. × 130

FIGURE 9.—Portion of large tumor in which capsule of spore mass at *right* has ruptured and permitted parasites to escape into host tissue. Alcoholic Bouin-Heidenhain. × 130

FIGURE 10.—Early changes in infected host tissue. Nuclei of migratory cells which have invaded infected connective tissue are rapidly proliferating. Portion of young cyst containing parasites appears at *left*. Formalin-Heidenhain. × 1300

FIGURE 11.—Final appearance of infected host tissue. Numerous small blood vessels are present and there is very little cellular proliferation. Alcoholic Bouin-Heidenhain. × 1300

Liver Lesions Produced by Hydatid-Like Cysts in an Elasmobranch, the Electric Ray, *Torpedo californica*[1]

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An electric ray (*Torpedo californica*) was discovered to have lobulated masses on the surface of the liver (figs. 1 and 2). Other organs revealed no pathology.

Grossly, the masses consisted of numerous grapelike cysts suspended and interconnected by connective tissue which was continuous with the liver capsule (figs. 3–6). The liver surrounding the cysts was highly inflamed and showed no neoplastic transformation (fig. 7). Although liver parenchyma was virtually obliterated due to replacement by blood cells and cysts, there was remarkably little fibrosis. The cysts may produce a chemical that inhibits fibrosis, as fibrosis would tend to seal off the cysts from vital contact with the surrounding liver tissue.

The cysts, particularly in gross appearance, resembled the hydatid cysts produced in higher vertebrates by tapeworms in the Order *Cyclophyllidea.* Other cyst-forming organisms, *viz.*, trematodes and microorganisms, were not observed in the ray liver lesions. Therefore, a cestode of obscure identity is considered to be the etiologic agent of the lesions.

The inner surfaces of most cysts appeared to have produced cell-like bodies which crowded into the cyst cavity and became necrotic (figs. 8 and 9). These bodies may represent tapeworm scolices that developed atypically because the ray is an abnormal host. In any event, the appearance of the cyst contents suggested that the proliferating bodies became necrotic and produced a granular deposit, or "sand," within the cyst (figs. 8, 10, and 11). These cysts may have been "sterile."

Cysts appeared to develop from a solid cluster of cells which accumulated fluid in the center (figs. 12–14). In most instances, the inner wall appeared to bear germinated bodies, but at least one large cyst did not produce any (fig. 15).

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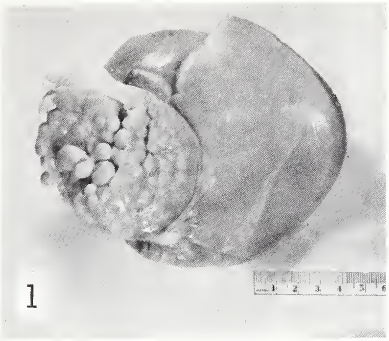
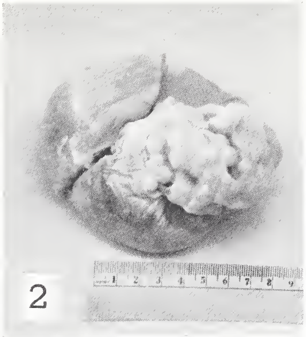
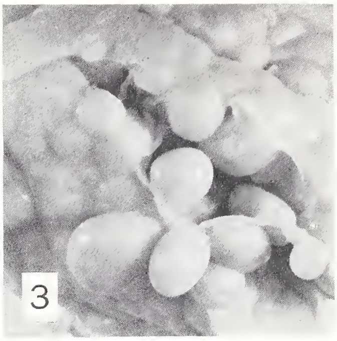
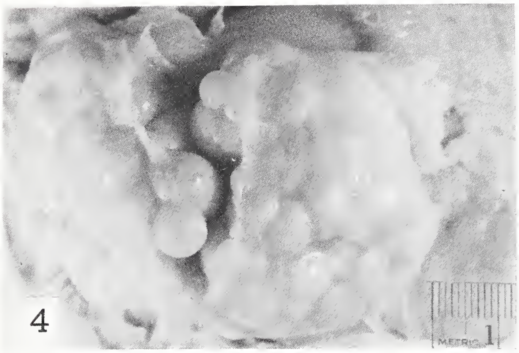
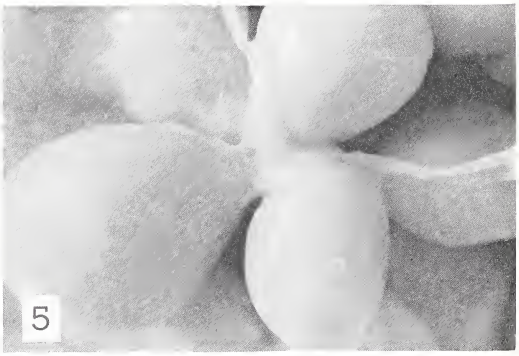
This is the first report of hydatid-like cysts in an elasmobranch, and possibly in the lower vertebrates. This is also, possibly, the first account of an elasmobranch containing proliferating cestode larvae, and therefore, of this group of fishes serving as an intermediate host in the life cycle of a tapeworm.

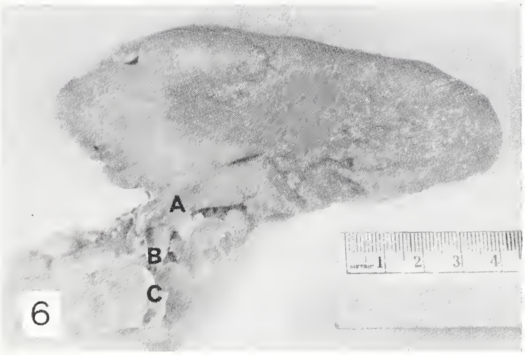


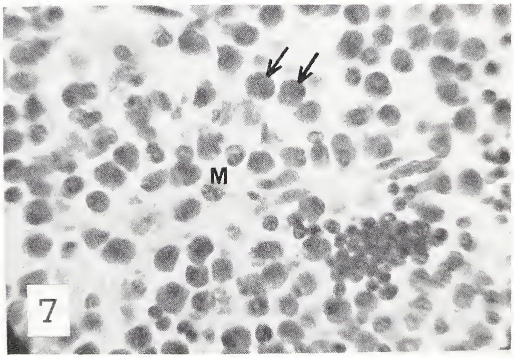












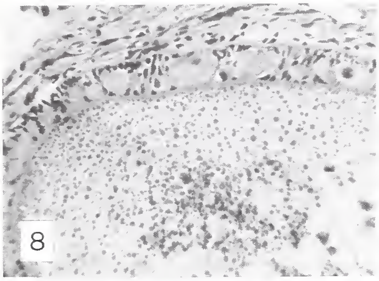
FIGURES 1 AND 2.—Lesions on right and left lobes of liver, respectively. *Note* connective tissue encapsulating both normal liver and lesion.

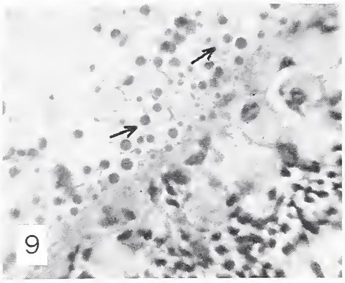
FIGURES 3 AND 4.—Entire lesions on right and left lobes of liver, respectively. Grapelike cysts are especially clear between torn edges of connective tissue capsule. Connective tissue penetrates between and interconnects cysts, sometimes forming strands.

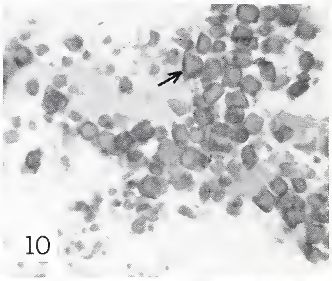
FIGURE 5.—Cysts interconnected by strands of connective tissue. × 2.7

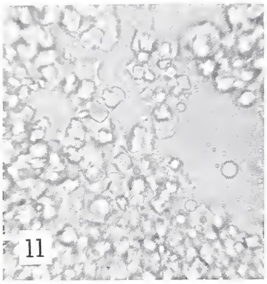
FIGURE 6.—Region of demarcation between abnormal and normal liver. *A* indicates normal liver; *B*, pathological liver; and *C*, sliced cysts.

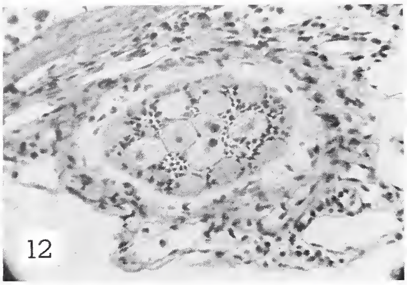
FIGURE 7.—Cellular composition of abnormal liver. Relatively small cells in clumps (*lower right*) are lymphocytes. Occasional monocyte is also visible (*M*), but most common blood cell type is granular leukocyte (*arrows*). Fibroblasts and connective tissue fibers are in *right center*. × 390

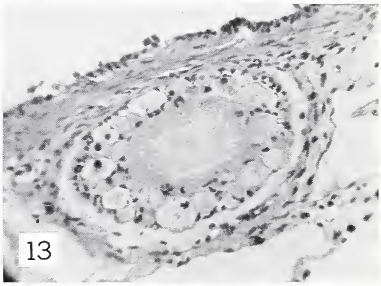


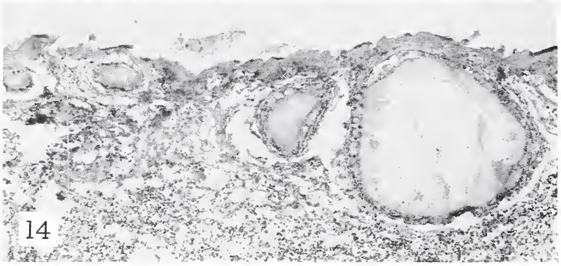












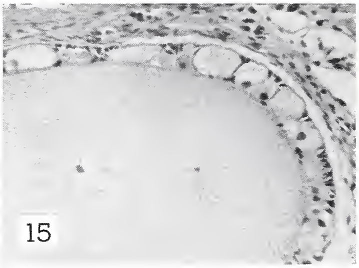


Figure 8.—Portion of cyst wall and cavity (*lower right*). Small bodies are on inner surface and are more crowded toward *center* of cyst where darkly stained particulate matter is abundant. × 125

Figure 9.—Bodies on inner surface of cyst wall resemble single cells, since some (*arrows*) seem to contain cytoplasm, nucleus, and nucleolus. × 290

Figures 10 and 11.—Histologic and squash preparations, respectively, of crystal-like particles in *center* of cyst. Most particles are approximately square, but an occasional one is helmet-shaped (fig. 10, *arrow*). Fluid is also present. × 290

Figures 12, 13, and 14.—Cysts appearing to be in successive stages of development

Figure 12.—Cluster of large, agranular cells and small, darkly stained cells. × 125

Figure 13.—A similar group of cells as seen in figure 12, but containing a central fluid-filled cavity. × 125

Figure 14.—Four cysts of different sizes. × 30

Figure 15.—Cyst with no cell-like bodies or particulate matter in central cavity. Compare with figure 8. × 125

Pigmented Tumor-Like Lesions in an Ophiuroid Echinoderm

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SUMMARY

During an investigation of the dark melanin-like pigment of *Ophiocomina nigra* (Echinodermata: Ophiuroidea), pigmented, epidermal, tumor-like lesions were seen. They consisted of ovoid, cellular nodules, 25–250 μ long, with pigment and epidermal cells as predominant cell types. Although their pathological nature is uncertain, they may be compared to pigmented nevi or melanotic tumors. Observations concerning growth by mitosis and invasion of peripheral epidermis suggest that the tumors may be true neoplasms.—Nat Cancer Inst Monogr 31: 255–261, 1969.

CONSIDERABLE ATTENTION has been given recently to the melanin-like pigments which are responsible for the dark color of the integument of certain echinoderms (*1*). The pigmented lesions that will be described here were noticed incidentally during an investigation into the nature of the dark pigment of the ophiuroid *Ophiocomina nigra* (Abildgaard), a species common and widespread in European waters. This research showed that the *Ophiocomina* pigment has the properties of a phaeomelanin, and that the pigment granules are cytologically localized in epidermal dendritic melanocytes, structurally analogous to those in amphibian skin (*2*). The tumor-like lesions carry large numbers of pigment granules disposed in morphologically abnormal melanocytes, as well as smaller quantities of lipofuscin granules. Consequently, the lesions may be compared in some respects to vertebrate melanomas. This appears to be the first reported occurrence of a spontaneous tumor-like lesion in the echinoderms, though supposed neoplastic tissue changes induced experimentally have been reported for some echinoderm species (*3*).

The *Ophiocomina* were collected by dredging from the Eddystone shell gravel off Plymouth, England, between August 1954 and June 1955. Unfortunately the lesions were not observed until the animals had been processed histologically, and it has not been possible to return to Plymouth to search for further specimens. Therefore the extant material consists of paraffin sections of seven adult *Ophiocomina* variously fixed and stained, which have been picked out of the slide series made during the fundamental study of the dark pigment.

Of 95 *Ophiocomina* examined histologically, 7 showed unequivocal tumor-like lesions. That is, 7.4% of the animals taken from their natural environment had spontaneous growths. This indicates that the natural incidence of lesions is within the range 5–10%, a rather high figure, suggesting further that the growth is of minor pathological significance to the individual in terms of health and survival in a natural population. The frequency of multiple lesions is also high. Of the 7 specimens, one had 3, three had 2, and three had 1 lesion.

The morphological sites for lesion development appear restricted to certain definite areas of the integument. These include the epidermis on, and especially between, the arm spines and the epidermis of the aboral surface of the disk and arms. The area between the arm spines seems especially favored, and the most massive growths occur here. They are absent from other areas of the integument, though the material is too limited to be categorical on this point. It may be significant that the most densely pigmented skin areas, and also those most exposed to light in nature, are the sites where lesions develop.

A typical lesion is illustrated in figures 1 and 2. Generally, the growths are noncalcified, ovoid nodules, ranging in diameter from 25–250 μ, and are dark because they contain pigment. Superficially, they appear to be dermal in situation, but appropriately stained sections show that they are clearly epidermal and that they lie entirely above the epidermal basement membrane (fig. 3), which delimits the basal portions of the growth. The basement membrane does not completely encapsulate the lesion, however, so that the lateral and superior surfaces have no clearly demarcated boundary. Thus, its cells are in contact with the normal epithelial cells immediately above and beside them (figs. 4 and 6). The normal external epithelium remains intact and is not obviously affected by the underlying growth.

At the cytological level, the nodules are composed of densely packed cells and appear completely devoid of connective tissue fibers. The predominant cell type is the epidermal melanocyte which, however, is abnormal in shape. The widely ramifying dendrites characteristic of the normal melanocyte are absent; instead, the cells are attentuated, comparable in shape to the mammalian fibroblast. They do, however, contain numerous pigment granules conferring a dark color on the whole lesion as seen in thick sections. A second common cell type is morphologically similar but contains light-brown, irregular granules giving the histochemical reactions of lipofuscin. This cell is interpreted as an abnormal form of the carotenoid-containing lipocyte, a normal constituent of *Ophiocomina* skin responsible for its red and yellow lipochrome colors (*4*). A third cell type is round, with a nonpigmented and an agranular cytoplasm. The exact nature of these clear cells is unknown, but they may represent invading coelomocytes and/or pathologically altered epithelial cells.

As indicated previously, there was a wide range of sizes in the lesions detected, despite the paucity of material. If they are observed in a sequence of increasing size, changes in the internal cellular pattern become obvious and can be interpreted as a developmental sequence correlating with lesion growth. These will be referred to as the early, intermediate, and late stages of development.

The early stage is a small, ovoid nodule causing a minor bulge in the epidermis (fig. 5). The overlying epithelium remains intact, however, as it does throughout the entire developmental sequence. At this stage, the cells appear to be almost exclusively one type, *i.e.*, abnormal, rounded melanocytes, and the growth is densely pigmented.

In the intermediate stage, the lesion is larger, the bulge more prominent, and its basal region presses on and compresses and distorts to a certain extent the underlying dermis (figs. 3 and 8). The distortion is probably due to pressure alone because there are no signs of invasion of the dermis by pathological cells, and the cytological picture of the dermis remains unchanged. Evidently the basement membrane is an effective barrier to cellular invasion from the lesion. The exception to dermal disruption occurs when the tumor overlies a skeletal plate (fig. 7). In this case, the supporting calcite is evidently strong enough to withstand the lesion's growth pressure, and the dermal structures remain unchanged. In this stage, abnormal melanocytes, lipocytes, and clear cells can be detected, and the dark pigmentation is less intense. Also at this stage, a number of pigmented fibroblast-like cells are visible which apparently have detached themselves from the main tumor mass. These cells are morphologically identical to embryonic pigment cells. They appear to be invading the surrounding tissue, and are most commonly observed in a position suggesting that they are moving outward between the basement membrane and the epidermal epithelium (fig. 4). In some instances, what appear to be small satellite lesions can be seen at a short distance from the main mass. They presumably have their origins from the colonial activities of the supposed embryonic pigment cells migrating outward from the focal lesion.

In the late stage of growth (fig. 8), the tumor is roughly divided into cortical and medullary regions. The medullary region is composed of cells that look highly undifferentiated and fibroblast-like. These are completely devoid of pigment and show frequent mitotic figures. The cortical regions are pigmented and have the same cell types and the same cytological pattern as noted for the intermediate stage. The abesence of pigment in the medulla results in the late-stage tumors being very lightly pigmented.

Mitotic figures are observable in all three stages, but they are most frequent in the medullary region of the late stage.

Although the developmental sequence is open to various interpretations, the following explanation of the events is offered. The origin of the lesion is due to an abnormality and subsequent hypertrophy of a locus of epidermal melanocytes. This involves a dedifferentiation of the melanocytes to a more or less embryonic condition. Further growth involves in a similar fashion the carotenoid-containing lipocytes and perhaps some of the epithelial cells, or wandering coelomocytes, or both. In this intermediate stage, migrant or invasive cells can be budded off, resulting in the establishment of peripheral colonies. In the later stages, dedifferentiation has proceeded to the point where the original cells have lost their melanogenetic ability, leaving a central or medullary zone of undifferentiated cells having the appearance of fibroblasts or embryonic cells in tissue culture.

Because of the limited material and the static, observational nature of the data, it is impossible to state unequivocally that this is a neoplasm. There are, however, three salient points that should be noted in regard to the potential neoplastic nature of the lesion. First, the earliest detectable stage has its focus or germinal origin in a cluster of abnormal pigment cells. These greatly resemble embryonic echinoderm melanocytes, indicating that the origin of the lesion is intimately related to a defect of the epidermal pigment cell population. Second, migrant cells occur peripheral to the main cellular mass and appear to move outward to establish satellite colonies at a distance. This phenomenon suggests a form of metastasis, or more properly a form of intraepidermal spread as seen in some junctional nevi. Third, the nodule grows by frequent cell divisions. This apparently trivial point is notable because echinoderm students have often commented on the surprising lack of mitotic figures in normal tissues. In echinoderm histology, mitosis in postmetamorphic animals is a rarely observed event, suggesting that in this case the mitotic activity of the lesion is one or more orders of magnitude greater than normal.

The involvement with, and apparent origin of, the growth in defective pigment cells indicates that it may be compared to a melanoma. On the other hand, in the absence of any evidence that the nodules are detrimental to the well-being of the animal, perhaps a more plausible interpretation is that the growths represent pigmented junctional nevi.

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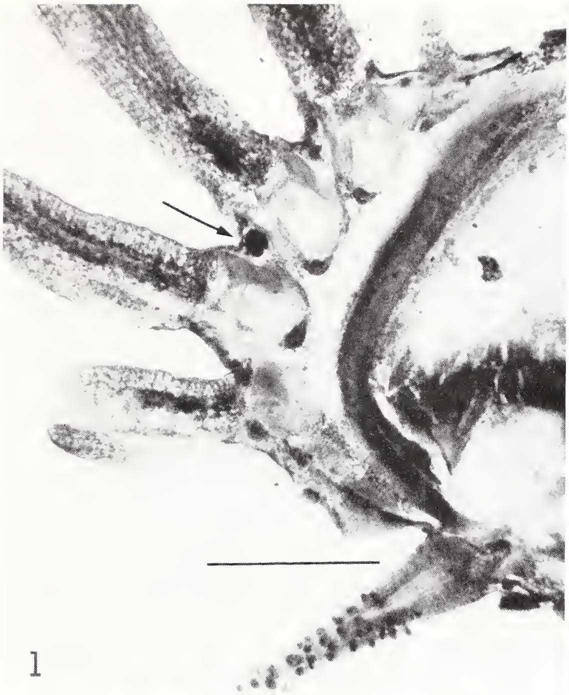

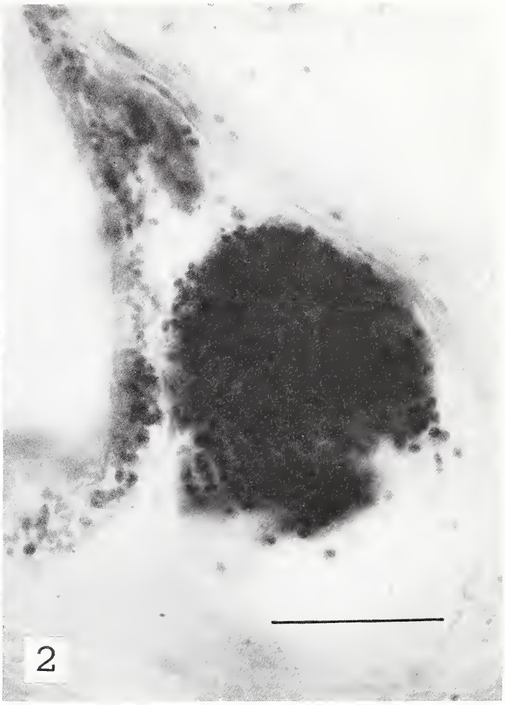

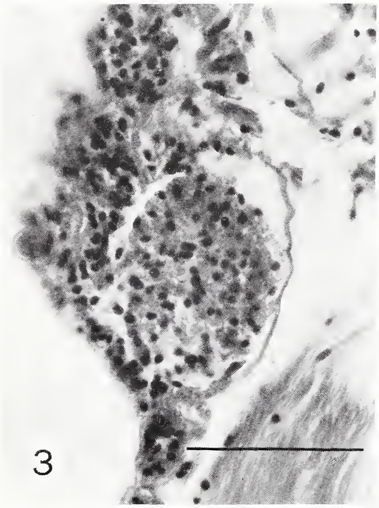

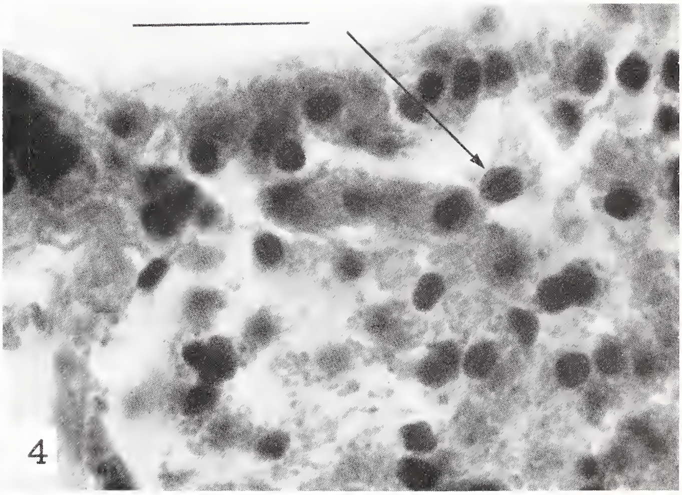

FIGURE 1.—Transverse section, 100 μ thick, of an *Ophiocomina* arm. *Arrow* points to an intermediate-stage tumor located between arm spines. Line measures 1 mm.

FIGURE 2.—Tumor seen in figure 1 shown at higher magnification. *Note* normal, intact external epithelium and apparent dermal position of tumor. Line measures 50 μ.

FIGURE 3.—A 10 μ section of an intermediate-stage tumor located (as in figs. 1 and 2) between arm spines. The thinner section and the stain display the basement membrane, proving the epidermal nature of the tumor. Line measures 70 μ.

FIGURE 4.—Oil-immersion photograph of an exterior-facing portion of figure 3. Pigment granules, which have been bleached, are evident in the cytoplasm. *Arrow* points to a presumptive invasive or migrating cell. Line measures 20 μ.

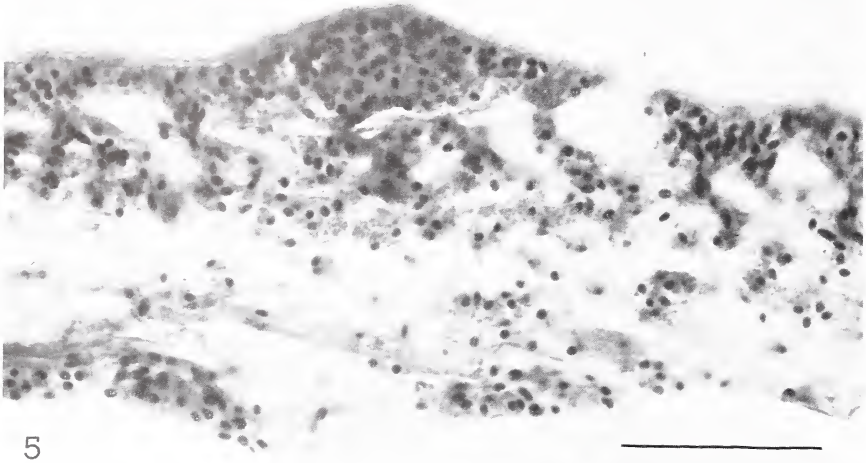

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FIGURE 5.—Early-stage tumor in aboral epidermis of an arm. The epidermal nature of the lesion and its encapsulation basally by basement membrane are evident. Line measures 60 μ.

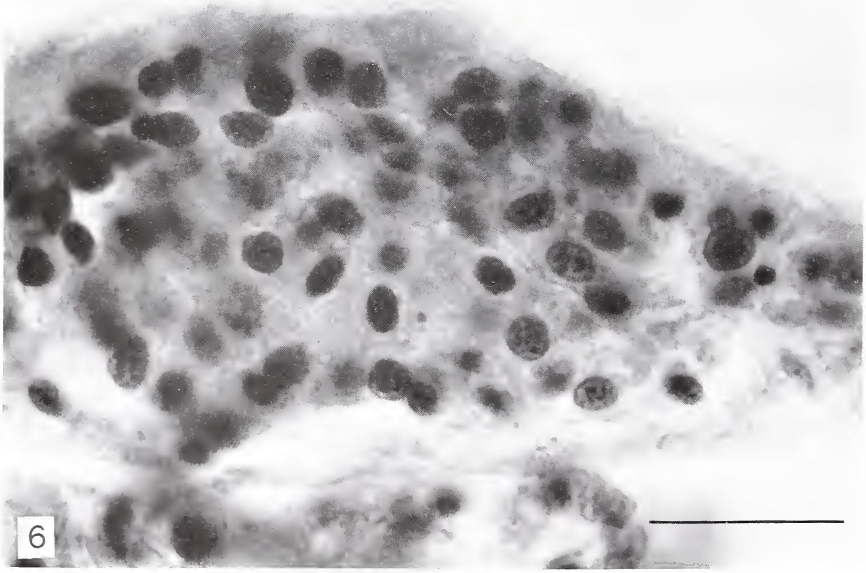

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FIGURE 6.—Oil-immersion photograph of figure 5. Intact external epithelium and basement membrane are obvious; rounded pigment cells are the only recognizable cellular elements within tumor. Line measures 21 μ.

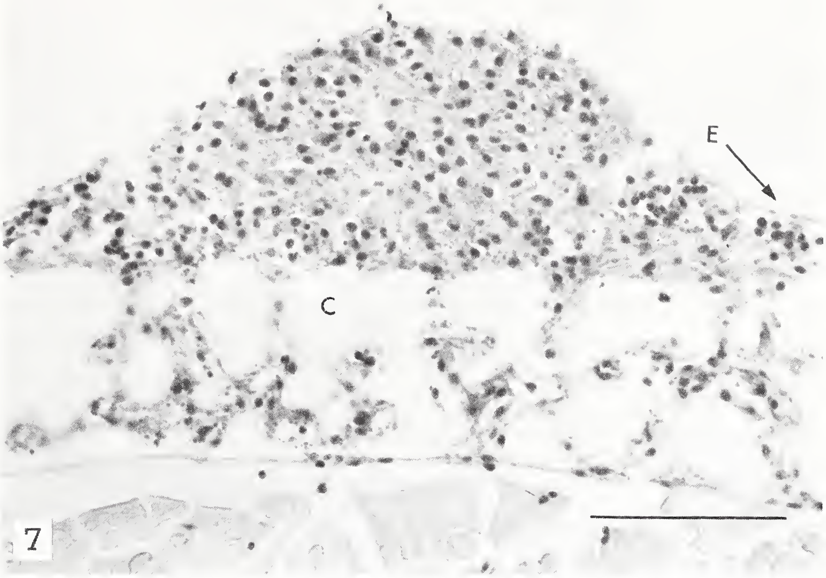

Figure 7.—Intermediate-stage tumor in epidermis over calcified dermis. Clear spaces were occupied in life by calcite, forming the skeletal plates of the integument. C, calcified dermis; E, epidermis. Line measures 80 μ.

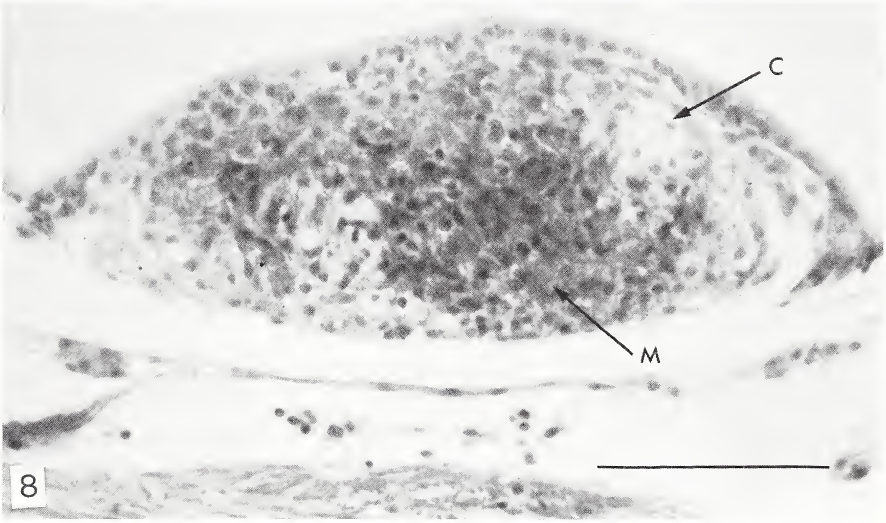

Figure 8.—Late-stage tumor showing cytological zonation into cortical (C) and medullary (M) regions. Line measures 80 μ.

Melanotic Tumors in *Drosophila melanogaster*

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SUMMARY

The tumorous strains of *Drosophila* differ from one another mainly by the genes responsible for the genetic control of the tumoral character. This control is generally polyfactorial, but the involved genes are not all of equal importance. There are major genes whose presence is indispensable, and modifying genes which, in the presence of the major genes, increase or diminish the probability of tumor development. The cytoplasm may also influence tumorigenesis. The incidence, multiplicity, and size of the tumors can be modified by different physical, chemical, and physiological factors. Each of these factors acts at a specific phase of tumorigenesis, and, according to the phase influenced, it affects different characteristics of tumorous populations. Two different events in the histogenesis of melanotic tumors can be distinguished in *Drosophila:* a neoplastic phenomenon consisting of the multiplication of abnormal hematocytes and an inflammatory event manifesting itself in encapsulation of the areas of abnormal growth by spindle-shaped blood cells. This encapsulation is responsible for the melanotic degeneration of tumors in *Drosophila.* The inhibition of the inflammatory reaction at the beginning of tumor formation by lowering of the temperature or blocking of the phagocytes allows the neoplastic cells to multiply and thus the neoplastic phenomenon can more easily be studied. The development of tumors after inoculation of blood or of acellular extracts from tumorous animals has been reported by several authors. The possibility of such an experimental transmission of tumors is very likely, but its definitive demonstration still needs careful histologic study.—**Nat Cancer Inst Monogr 31: 263–275, 1969.**

MELANOTIC TUMORS in *Drosophila* are malformations occurring as black masses of irregular shape. While they most often are located in the abdomen of the insect, they can be seen occasionally in the thorax and infrequently in the head and legs (fig. 1).

TUMOROUS STRAINS

The tumor-bearing individuals appear from time to time in laboratory stocks and are found also in natural populations (*1–4*).

Many tumorous strains characterized by a high penetrance of the tumor phenotype were constituted by selection. In a recent review, Harshbarger and Taylor (*5*) reported that the tumorous strains of *Drosophila melanogaster* at present number 55. These strains differ from one another by the proportion of tumor-bearing individuals, the mean number of tumors per individual, the time of tumor appearance, and, especially, by the genes responsible for their hereditary transmission.

The genetic control of *Drosophila's* melanotic tumors is generally polyfactorial (*6–11*). From the importance of their effect, one can distinguish the major genes, whose presence is indispensable for tumor development, and the modifying genes, which, in the presence of the major genes, influence the frequency of the tumors.

The same tumoral gene may act as a semidominant or as a recessive, depending on the genetic background with which it is associated (*8*). It is also reported that cytoplasm plays a role in the control of tumoral character (*12*, *13*).

A statistical study of genetically homogeneous populations has shown a Poisson's distribution of tumors in larvae and young pupae. However, in old pupae and in imagoes, the distribution of tumors may no longer follow this theoretical regularity, and the number of tumors becomes lower than in young pupae (*8*).

The decrease in number of tumors in imagoes was also reported in two other publications (*14*, *15*). A regression of melanotic tumors in pupae having never been observed, the reason for the decrease in the number of the tumors and for their irregular statistical distribution in imagoes may be found in the fusion with neighboring tumors when they grow larger.

EXPERIMENTAL MODIFICATIONS ON TUMORIGENESIS

Many factors can influence incidence, multiplicity, and statistical distribution of tumors in *Drosophila.*

Nutrition

Several authors, working on different tumorous strains, have shown that starvation reduces the size and the incidence of tumors (*8*, *10*, *16–18*), if the food deficiency begins before the first appearance of tumor cells and if it persists until pupation. Transfer of larvae to a richer medium reverses this effect (*8*). Starvation seems mainly to influence growth of the tumors; its effect on their size can be observed, even without any decrease in their number. Reduction of tumor frequency probably results from a precocious and complete stoppage of the growth of certain tumors.

The decrease in tumor frequency in starved larvae is evidenced both by lower tumor incidence and fewer tumors per individual. The statistical distribution of tumors remains in accord with Poisson's formula.

The composition of the larval food also influences tumorigenesis in *Drosophila.* However, research workers who have studied the action of different amino acids and several vitamins on the development of tumors in this animal do not agree in their conclusions (*16–29*). This divergence in results may be explained by the possible differences in the nutritive needs of the tumorous strains used (*30*, *31*). The basic medium used in these experiments has not always been of the same composition. It is not only the presence or absence of one product in the diet that is important but also the balance between the different components (*32*). The period during which tumor incidence can be influenced by the composition of the food occurs, according to Burnet and Sang (*33*), at the end of the second larval instar. The action of some organic compounds that are not usually present in the larval food has also been studied. Among these chemical compounds, the salts of phthalic acid decrease the tumor incidence in larvae that absorb them; however, in the descendants of the treated larvae, on the contrary, the tumor incidence increases progressively during several generations and becomes higher than in control populations (*34*, *35*). Some mineral salts (silver nitrate, sodium fluoride) increase tumor incidence (*36*, *37*).

Temperature

The influence of temperature on tumor development in *Drosophila* has been reported by many workers (*10*, *38–40*). The curves of tumor incidence as a function of temperature differ between strains, but generally the highest temperature compatible with the achievement of metamorphosis decreases the incidence of tumors.

The following conclusions have been drawn from the results obtained with larvae of two different tumorous strains: *cl tu*^{48a} and *tu*^{48a} *vg bw* (*8*) : 1) The period during which tumorigenesis can be affected by a temperature of 29–30°C is in the second half of the larval life. Heating of larvae less than 48 hours old does not give any significant result. 2) The age at which temperature ceases to have any effect on tumorigenesis depends on the genetic constitution of the larvae. 3) Between the beginning and end of the receptive period, the heating effect is independent of the larva's age. 4) There exists a quantitative relationship between the duration of heating and the effect produced. 5) The heating acts according to the law of "all or none," *i.e.*, it inhibits the formation of *all* tumors in an individual or it does not suppress *any* of them. In this way, the statistical distribution of tumors is changed. 6) Heating does not change the size of the tumors.

The absence of any effect of heating on growth of the tumors and the coincidence of the temperature-sensitive period with that during which histologic examination reveals the appearance of tumors in larvae favor the idea that heating affects some initial stage of tumorigenesis. It does not interfere, however, at the site of the tumor itself, but must act at the level of the individual organization, creating a physiologic condition that is incompatible with the initiation of tumors.

The lowering of the temperature below 7°C, which arrests the activity of the larvae, increases not only the incidence but also the multiplicity and the size of the tumors, as was shown by experiments with larvae of the *cl tu*$^{48\,a}$ strain.

The importance of the effect produced by cooling depends both on temperature and duration of the treatment. The period during which the larva is sensitive to cooling almost coincides with that of sensitivity to heating (*41*).

Ionizing Radiations

X irradiation of embryos or young larvae of tumorous strains increases considerably the incidence of tumors (*33*, *42–47*). The same doses of radiation applied to the embryos of nontumorous strains seldom induced the formation of melanotic masses resembling tumors. Plaine (*46*) and Burnet and Sang (*33*) found that sensitivity to the tumorigenic action of X rays decreases quickly after the end of the first larval instar. It was shown, however, in experiments performed with *cl tu*$^{48\,a}$ larvae, that the tumorigenic effect of X rays persists during the whole period of larval life. This effect, while not disappearing completely, declines abruptly after the first larval moult, and a new sudden fall in sensitivity is also observed after the second larval moult (*48*). However, larvae from irradiated gametes do not develop more tumors than nonirradiated controls.

With the increase in radiation dose, more tumors appear; then, after reaching a maximum, the number remains steady, and finally decreases when the dosage reaches a certain limit. The dose necessary for inversion of the radiation effect on the tumorigenesis varies between 1500 and 3000 R, depending on the ontogenetic stage of the irradiated insects and the strain to which they belong (*42*, *48*). Irradiation raises the incidence of tumors, increases their multiplicity and size, and causes their early formation (*47*). The effect of irradiation is much less when performed in the absence of oxygen—in a nitrogen atmosphere (*44*, *45*). Oxygen alone, without irradiation, has little influence on tumor development (*45*).

Beta irradiation, from tritiated nucleosides or tritiated amino acids incorporated by the larvae, produces the same effect as external X irradiation (*49*).

Pupation Hormone

Burdette (*50*) and Oster (*51*) hypothesized that the degeneration of cells composing melanotic tumors occurring toward the end of the larval life could be due to the same cause that provokes lysis of larval tissues during metamorphosis. To verify this hypothesis, they introduced into the genotype of tumorous flies genes to lengthen the larval period. The larvae, in which pupation was consequently delayed, developed more tumors, and these results seemed to confirm this hypothesis. However, in other experiments, ligation of the third instar larvae behind the ring gland gave contradictory results: Whereas Burdette (*52*) observed an increase in the number of tumors in the posterior part of the body, deprived by ligation of all influence of hormone-secreting centers, Rizki (*53*) found a decrease.

Of course, a slight difference in the age of the larvae at the time of ligation may change the results of an experiment. But it is difficult to accept a direct link between metamorphosis and the degeneration of tumors in *Drosophila.* This degeneration does not coincide necessarily with the beginning of nymphosis, but can also precede or follow it. Moreover, the responsibility of the humoral factor in the degeneration of tumors seems unlikely because there can be found, in the same individual, tumors that are completely melanized, containing almost no cellular elements, and others in which melanization has not yet begun.

HISTOGENESIS OF MELANOTIC TUMORS

The histologic picture of the successive stages of the development of melanotic tumors is alike in the larvae of different tumorous strains. In all of them, two kinds of cells in the tumors can always be distinguished: round or polygonal and spindle-shaped (*54–61*). The tumors originate from a group of a few polygonal cells (fig. 2). These cells soon become surrounded by the spindle cells (fig. 3), which will later form the big mass of the tumor. The polygonal cells appear then isolated or dispersed in small groups, in a kind of stroma of more or less loose texture formed by the spindle cells. At this stage of its development, the tumor often begins to spread between the cells of the fat body, sometimes surrounding entire lobes of this tissue (fig. 4).

At a later stage in its development, the cells of the tumor become pigmented with melanin (*62*, *63*). The appearance of this pigment coincides with the degeneration of tumor cells. The degeneration affects also the fat cells embedded in the tumor.

Oftedal (*57*) has reported that the tumor cells are hematocytes; several facts corroborate this opinion: 1) A constant relationship seems to exist between the tumorous character of a strain and the high frequency of large hematocytes in the larval hemolymph (*58*, *59*, *64–66*). These hematocytes, as described by Strunge, Gigante, and Bernhard (*67*), have also a clearly pathological character. 2) Occasionally one can observe tumoral transformation of the lymph gland (*48*, *68*, *76*). 3) The X-ray doses responsible for the rise in tumor incidence determine often, if not always, hypertrophy of the lymph glands, whereas larger doses which inhibit tumor development cause atrophy of the lymph glands (*69*). 4) Tumor cells can phagocytize foreign particles introduced in the hemocoel of the larva (fig. 5).

PATHOLOGIC SIGNIFICANCE OF MELANOTIC TUMORS IN *DROSOPHILA*

If a unanimity of opinion exists at present about the origin of cells which compose the melanotic tumors of *Drosophila*, the pathologic significance of these malformations remains still debatable. Whereas some workers consider them an inflammatory or degenerative manifestation (*56–58*, *61*, *70*) and designate them "pseudotumors" or "granuloma," others believe that a neoplastic process may contribute to their formation (*55*, *59*, *60*, *71*, *72*).

The former base their interpretation on the fact that mitotic figures are not found in the tumors and these tumors degenerate sooner or later. The very histologic aspect of the tumors resembles, by the predominance of spindle cells, nodules that are commonly formed in insects around foreign bodies, parasites, or during certain infections.

In favor of the neoplastic hypothesis is the invasive tendency of the tumor, its volume (*60*), and perhaps also its ability to survive *in vitro*, as reported by Kuroda and Tamura (*73*) and Friedman and Burton (*74*).

The choice between these two interpretations depends chiefly on the answer to the question: Do the tumor cells multiply *in situ*? Two series of experiments in which the immunologic reaction of larvae has been diminished or inhibited at the time of tumor formation have shown: *a*) that the polygonal cells and spindle-shaped cells of the tumor are not of the same pathologic significance, and *b*) that the polygonal cells are able to multiply actively.

In the first experiment, the phagocytes of the larvae were blocked by heavy injection of India ink before the formation of the tumors (*59*). In the second experiment, the immunologic reaction was retarded by placing the larvae at the temperature of 4°C (*75*). In both cases, by histologic examination, tumors were found that were quite exclusively formed of polygonal cells; these cells were much more numerous than in the tumors developed in the controls (figs. 6 and 7). The polygonal cells were, therefore, neoplastic. Because of their size, they were thought to be cells derived from the largest hematocytes, whose presence in the hemolymph is characteristic of the tumorous strains. The multiplication of neoplastic cells may be stopped by their encapsulation. The spindle cells responsible for this encapsulation are probably normal blood cells. Their presence in the hemocoel is not specific for the larvae of tumorous strains, as reported by Oftedal (*56*, *57*); they also can be found in the larvae of nontumorous strains (*76*).

Thus, two different events contribute to the formation of melanotic tumors in *Drosophila:* One of them is neoplastic, the other inflammatory. The degeneration of the cells of the tumor and its melanization are the result of the intense inflammatory reaction to the neoplastic cells. The existence of an antagonism between the neoplastic process and immunologic reaction in *Drosophila* has already been suggested by El Shatoury (*77*), who attributed the cause of the degeneration of tumorous cells to a reaction of humoral immunity.

EXPERIMENTAL TRANSMISSION OF MELANOTIC TUMORS IN *DROSOPHILA*

The possibility of experimental transmission of melanotic tumors in *Drosophila* has been claimed by many authors. The grafts of tumors (*54*, *78*) and the inoculation of blood (*79*) or of acellular extract of tumorous larvae (*74*, *80–85*) have resulted in the formation in inoculated hosts of black masses morphologically resembling the tumors. However, because of the important role played by the inflammatory reaction in the formation of the melanotic tumors in *Drosophila*, it is difficult to interpret these results. Certainly some, at least, of the pigmented masses found in the body of the inoculated insects are not tumors, but patches of necrotized tissue or nodules formed around the inoculum in the absence of all neoplastic reaction. This would explain not only the inconsistency of the results obtained after an injection of tumorous larvae extracts (*86*), but also the formation of similar black masses after injection of nontumorous material (*87*, *88*).

Nevertheless, the possibility of experimental transmission of tumors by inoculation of a tumor-inducing factor in *Drosophila* remains very likely (fig. 8). The tumor-inducing factor has also been postulated to explain the inhibition of tumorigenesis at the temperature of 30°C (*89*). This factor might be a physiological product of larval metabolism, as Rizki believes (*53*), or it might possess the properties of a virus (*82*), or finally, it might be an autoreproducible particle having the characteristics of an episome, according to Barigozzi (*90*).

An electron microscope study by Bairati (*91*) revealed virus-like particles in the cells of the lymph gland in larvae of the *tu B 3* strain. However, such particles were not found in the larvae of other tumorous stocks (*67*). Whatever may be its cause, one must recognize that an actual neoplastic process originates melanotic tumor formation in *Drosophila*. The benign character of the malformations is explained by the encapsulation of tumorous cells. This defense reaction was also observed in tumors of different origin developed in other insect species. The penetration of hematocytes in the neoplastic proliferation area has been described by Pflugfelder (*92*) in *Carausius* (*Dixippus*) *morosus* tumors that developed after extirpation of *Corpora allata* and by Matz (*93*) in the tumors of the gut produced in *Locusta migratoria* by the section of a sympathetic nerve.

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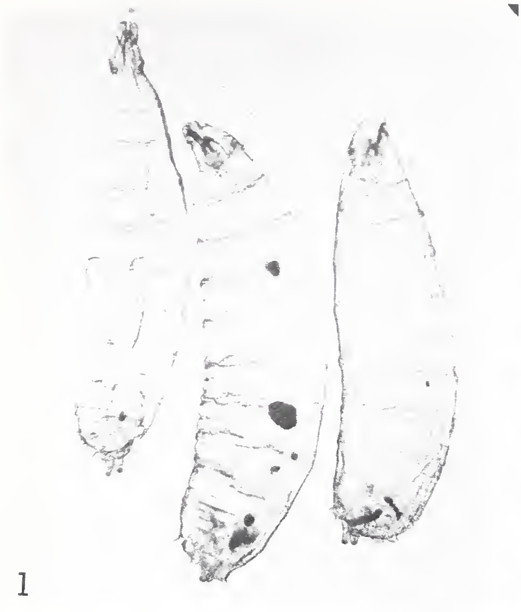

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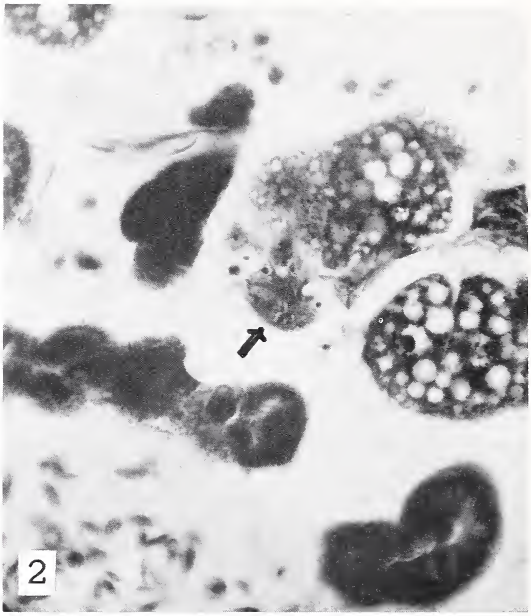

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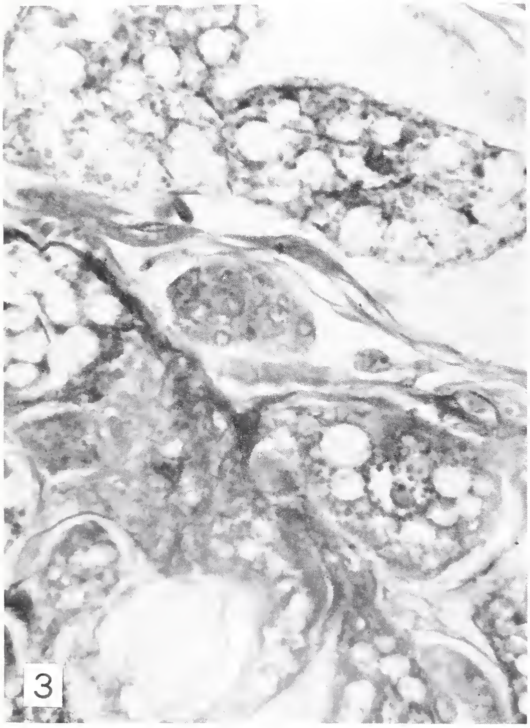

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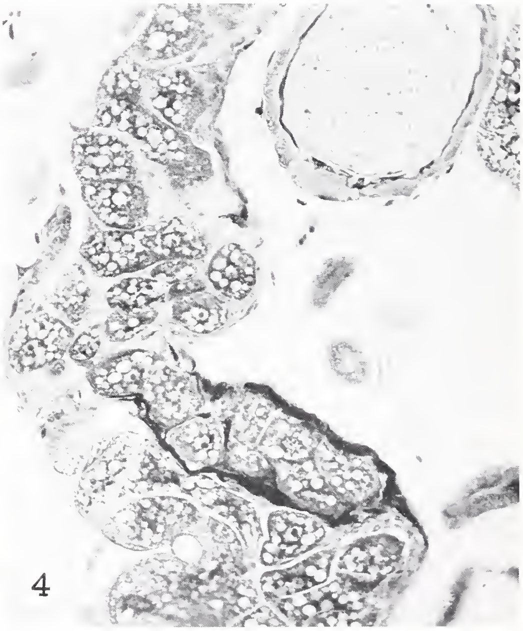

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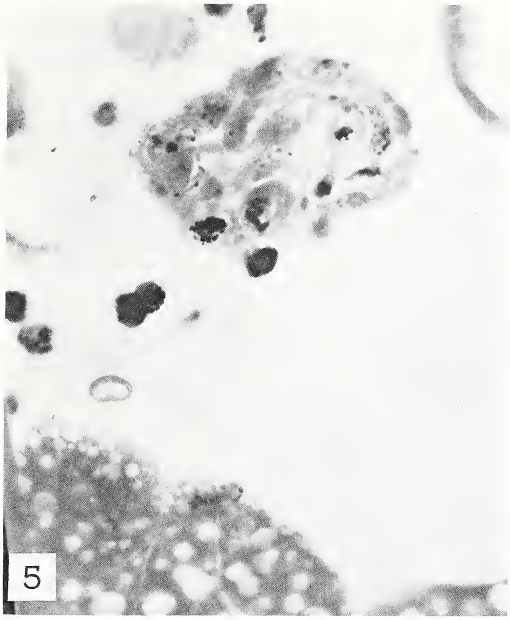
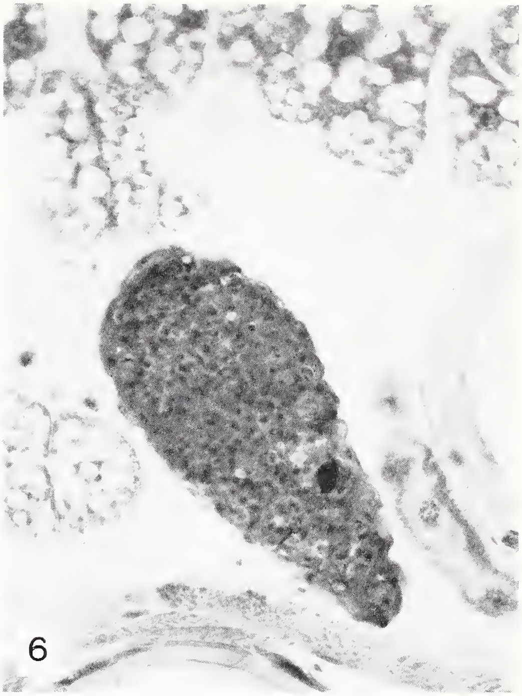
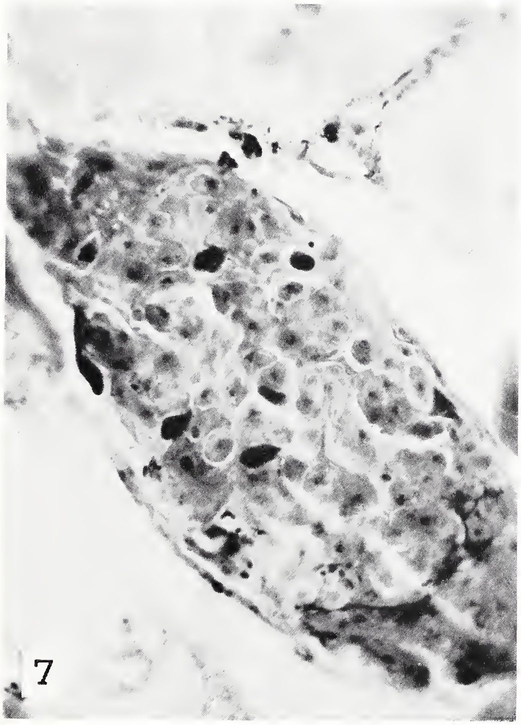
FIGURE 1.—Late third instar *tu*^{48a} *rg bw* tumorous larvae.
FIGURE 2.—Beginning of tumor development: a few polygonal cells.
FIGURE 3.—Group of polygonal cells surrounded by spindle-shaped cells.
FIGURE 4.—Partially melanized tumor invading fat body.







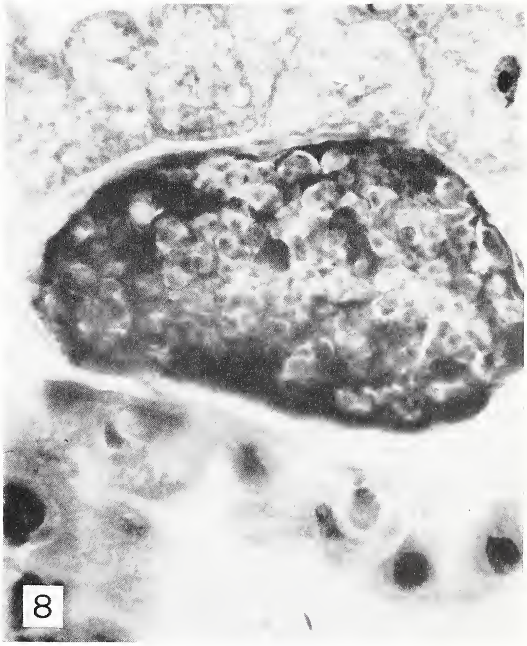


FIGURE 5.—Phagocytosis by tumor cells 5 hours after inoculation of India ink.

FIGURE 6.—Tumor showing only polygonal cells in a *tu*$^{48\,a}$ *vg bw* larva 94 ± 2 hours old, fixed 23 hours after inoculation of India ink.

FIGURE 7.—Tumor of origin similar to that in figure 6, but at a higher magnification.

FIGURE 8.—Tumor developed in a larva from nontumorous strain, fixed 43 hours after simultaneous inoculation of an acellular extract from *cl tu*$^{48\,a}$ larvae and India ink.

Genetic Control of Melanotic Tumors in *Drosophila*

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SUMMARY

The problem of how to classify insect "melanotic tumors" composed of blood or fat cells is considered and the term "pseudotumor" is proposed. The melanization process, the difference between spontaneous and induced pseudotumors, and the effects of pseudotumors on fitness are described and discussed. The data lead to the conclusion that these lesions are generally harmless and frequent. A basic mendelian mechanism of transmission of pseudotumors is demonstrated, which seldom is controlled by a single gene, but by at least two polygenic systems. In cases analyzed in detail, at least one polygenic system controls the behavior of the lymph organ and the lamellocytes. Another system (mostly in the second chromosome) is generally referred to as *tu*-gene and is believed to control melanization. Besides this mode of transmission, clear indications are found of the existence of extrachromosomal entities, transmitted through both gametes. Pseudotumors are interpreted as a response to external or internal stimuli. The action of the *tu*-genes is discussed, since possibly they control either the melanin formation or the stimulating agent, against which the lamellocytes and melanin formation system react.—Nat Cancer Inst Monogr 31: 277–290, 1969.

EVEN THOUGH the main task of this paper is to discuss the genetic control of the melanotic tumors in *Drosophila*, to provide some background for interpreting their significance, I think it useful to describe typical traits of these tumors. Three topics are briefly considered: 1) melanization, 2) morphological and structural identity between spontaneous (hereditary) and induced (nonhereditary) tumors, and 3) influence of tumors on fitness.

First, a statement on nomenclature: Among the different names proposed for proper classification of the so-called melanotic tumors of insects, I believe *tumor* is most inappropriate and can lead to confusion between these formations and neoplasms; *pseudotumor* is better. However, *tumor* will be used throughout this paper as an abbreviation, without the significance of cancer or neoplasm.

1) *Melanization.*—In almost all tumors described in *Drosophila*, the black masses consist initially of clumps of blood cells (lamellocytes). Mel-

anin is deposited in the intercelluar spaces, but, since no evidence exists that it is produced by the lamellocytes (*1*), Rizki's hypothesis (*2*) remains unproved. In a few cases, melanization occurs in fat cells as, for example, in *Freckled* (*Frd*) (*3*). This stock also contains a secondary melanization of the pericardial cells, due to storage of melanotic debris through phagocytosis. Ovarian tumors (*4*) based on uncontrolled cell proliferation (probably true tumors), and not on melanization, are not considered here, nor are the stocks described as malignant by El Shatoury (*5*).

2) *Morphological and structural identity between spontaneous and induced melanotic tumors.*—Castiglioni and Beati (*6*) and Röhrborn (*7*) showed this identity. The prerequisites for a melanotic response to injection of suitable fluids (hemolymph, etc.) are the existence of a rather high frequency of lamellocytes (3–4%) and of a dissolved lymph organ in the larval stage, which, in tumor-free stocks, disappears only during pupation.

3) *Fitness in tumorous and nontumorous genetically similar stocks.*—The elements of fitness considered by Di Pasquale and Koref (*8*) were fecundity, viability, sterility, and rate of development. No difference was found between one tumorous ($tu\ B_3$) and one nontumorous stock (Varese), but, in another case, heterozygotes laid more eggs than *tu*/*tu* and +/+ homozygotes. Sometimes fitness was lower in *tu* homozygotes than in +/+ homozygotes. Lethality due to tumors is exceptional.

THE TRANSMISSION OF MELANOTIC TUMORS IN GENERAL

Occurrence of Melanotic Tumors in Natural Populations

Tumors occur in nearly all stocks of the *Drosophila* species, but their incidence is seldom higher than 2–4%. Few investigators (*9*, *10*) paid attention to the occurrence of tumors in wild flies, though this information is important for an interpretation of the phenomenon in general. The observations by Di Pasquale and Koref (*11*), which may be cited as the most analytical in this connection, refer to two collections of flies made near Palermo, Sicily, in August and September 1957.

Among the 16 *D. melanogaster* and the 46 *D. simulans* of the first collection, and the 21 *D. melanogaster* and the 75 *D. simulans* of the second, a tumor incidence of 10 and of 28.2%, respectively, was found. From the first generation born in captivity, only a minority of offspring were tumor free (table 1). With 36 lines of *D. melanogaster* and 94 of *D. simulans* kept in culture, after 12 generations of brother × sister matings, a peculiar fact was observed: Tumor frequency was constant in some lines, regularly decreasing or increasing in others, and very irregular in a fourth group. The percentages of the four groups, from an analysis of variance, are given in table 2. We shall return to this subject later.

The great number of tumor lines derived from the same wild sample raises the problem of their genetic diversity. The answer is given in table 3, which shows the frequencies of interline crosses. The very low tumor incidence indicates that the genotypes of the 5 lines considered are recessive and differ as regards *D. melanogaster.* In *D. simulans* (table 4) the conclusions are not so clear. The rather high tumor frequencies (although with a broad variation among the 5 repetitions of each cross, of which the extreme values are given) may be interpreted either as dominance of one genotype or as a basic uniformity of the genotype sample that had been considered. No further analysis has been undertaken.

Many other data point to a very high variation of tumor-controlling genotypes, whose occurrence is most diffuse in wild populations.

Different Aspects of the Genetic Control of the Tumorous Phenotype

The genetically controlled differences between tumorous stocks are manifold. One of these relates to the moment, in the life cycle of the fly, when the pseudotumors appear. Nine stocks (4 wild and 5 mutants) have been scored for tumors [table 5 (*12*)] during larval life. The development is given in terms of total larval length, and the melanotic masses are recorded for each length interval. The onset is sometimes early and sometimes late; stock differences are clear and stable in each case. During the larval stages, tumorous individuals become more frequent; in the pupa and in the imago their number remains stable. Only *tu So* ^{C} requires special mention, since a decrease toward the end of the larval stage is observed. This may mean a differential survival rate between tumorous and tumor-free individuals. *Frd* (*3*) is the only case known to me of late onset during pupal stage. During the adult stage, practically no changes occur.

The number of tumors per larva is also genotypically controlled (*12*). In table 6 three stocks are considered (*tu* A_2, *tu* B_3, and *melanotic e* 144), two of them after a genome manipulation consisting of chromosome replacements (*see* later). The percentage of flies having more than 4 tumors per individual is different at least in 3 cases.

The anatomical localization of tumors in the adult is stock specific. Most stocks carry abdominal tumors. Localization in the thorax is rare and in the head even rarer. A unique pattern is shown by *Frd*, where melanization is extremely diffuse, owing to its relationship with the fat cells.

REFINED ANALYSIS OF THE GENOTYPE IN SOME TUMOROUS STOCKS

Chromosomal Localization and Mapping

This proceeds through two steps: identification of the chromosome or chromosomes involved and mapping within the chromosome (*13*).

Table 7 shows that in most cases the second chromosome only, or predominantly, is involved; in a smaller group the second and third or all pairs are equally active. For *tu* A_2, *tu* B_3, *tu* C_4, *tu D*, *melanotic e* 144,

Table 1. Frequency of pseudotumors in wild flies collected near Palermo, Sicily (*11*)

	Percent of lines having:								Total lines	Tumorous lines (%)
Tumors (%)	0	0.1–5	5.1–10	10.1–15	15.1–20	20.1–25	25.1–30	30.1–35		
D. melanogaster										
First collection	18. 8	37. 5	37. 5	—	6. 3	—	—	—	16	13 (81. 2)
Second collection	52. 4	38. 1	—	—	9. 5	—	—	—	21	10 (47. 6)
D. simulans										
First collection	15. 4	66. 7	15. 5	2. 6	—	—	—	—	40	34 (85. 4)
Second collection	12. 2	25. 7	25. 7	16. 2	8. 1	4. 1	5. 4	2. 7	74	65 (87. 8)

TABLE 2.—Behavior of tumor frequencies in inbred lines derived from wild flies (*11*)

	Percent of lines in which tumor frequency				No tumors
	Increased	Decreased	Behaved irregularly	Remained constant	
D. melanogaster					
First collection	12. 5	12. 5		75. 0	16
Second collection			15. 0	85. 0	20
D. simulans					
First collection	7. 5	5. 0	15. 0	72. 5	40
Second collection	5. 6	7. 4	1. 9	85. 3	54

TABLE 3.—Tumor frequencies in crosses within and between inbred lines from wild flies (*D. melanogaster*) (*11*)

Within line		Between lines			
Line	Percent *tu*	Lines crossed	Percent *tu*	Lines crossed	Percent *tu*
6	2. 1	6 × 7	0. 3	7 × 102	0. 2
7	4. 6	6 × 52	0. 6	7 × 154	0. 8
154	6. 0	6 × 102	0. 1	52 × 102	0. 8
52	65. 8	6 × 154	1. 1	52 × 154	1. 9
102	6. 8	7 × 52	1. 1	102 × 154	0. 9

TABLE 4.—Tumor frequencies in crosses within and between inbred lines from wild flies (*D. simulans*) (*11*)

Within lines			Between lines					
	Percent *tu*			Percent *tu*			Percent *tu*	
	a*	b*		a*	b*		a*	b*
37	73. 6	55. 8	42 × 37	45. 0	89. 2	37	2. 7	43. 7
42	46. 6	20. 1	42 × 148	29. 3	85. 7	42	5. 0	28. 4
148	48. 1	27. 1	42 × 156	46. 7	59. 2	148	0. 0	22. 0
156	16. 7	30. 1	37 × 148	37. 3	80. 2	156	7. 5	14. 1
			37 × 156	20. 7	40. 6			

*Tumor incidence in two subsequent generations.

TABLE 5.—Differences in tumor manifestation during larval development in different tumorous stocks (*12*)

Stock	Length (mm)							
	0.5–1.0	1.0–1.5	1.5–2.0	2.0–2.5	2.5–3.0	3.0–3.5	3.5–4.0	4.0–4.5
tu A_2	0	0	0	0	2	15	53	89
tu B_3	0	0	0	2	15	37	58	87
tu C_4	0	0	0	0	15	24	55	83
tu D	0	0	0	0	8	25	52	60
tu-w	7	8	9	9	9	18	14	15
tu-y Hw	2	3	5	4	8	10	8	13
tu-NB^{-s}	6	6	7	5	7	12	23	18
tu-mwh	1	3	3	6	9	32	38	38
tu-So^{C}	8	17	20	33	22	9	16	5

TABLE 6.—Tumor frequency per larva (*12*): Percentage of larvae with more than 4 tumors

Stock	Larval length (mm)	
	2.0–2.5	4.5–5.0
tu A_2 (original stock)	—	—
tu A_2 (second chromosome)	—	7%
tu B_3 (original stock)	—	28%
tu B_3 (second chromosome)	—	1%
melanotic e 144	—	—

TABLE 7.—Effect of the main chromosome pairs on tumor frequency in different stocks (*13*)

Chromosome*			Stock	Authors
1	2	3		
.	+	.	*tu* A_2	Barigozzi and Di Pasquale
.	+	.	*tu* B_3	″ ″ ″
+	+	+	*tu* C_4	″ ″ ″
.	+	.	*tu D*	″ ″ ″
.	+	.	*melanotic e* 144	Barigozzi, Castiglioni, and Di Pasquale
.	+	+	*Aspra* 52	Di Pasquale
.	+	.	*Aspra* 69	″
+	—	—	1–*m*	El Shatoury
—	+	—	—	Ghelelovitch
—	+	—	*tu-er*	Glass
+	+	—	1-*nd*	El Shatoury
—	+	—	*bw-tu*	Hartung
—	+	—	tu^{48}_{s}	Herskowitz and Burdette
—	+	—	—	Russell
—	+	—	—	Stark and Bridges
—	+	+	*tu* 1	Wilson
—	+	+	*tu* 2	″

* + means a frequency >10%; . means a frequency <10%; — means no tumors.

Aspra 52, and *Aspra* 69, all possible homozygous combinations of *tu* and non-*tu* chromosomes have been studied. This technique has made it possible to determine what weight to attribute to each chromosome pair.

Stocks *tu* A_2, *tu* B_3, and *melanotic e* 144 have been analyzed and the main findings are in table 8. As I have said, prerequisites for the production of melanotic masses are a minimum amount of lamellocytes and their delivery from the lymph organ, through its disintegration. Both phenomena are controlled by more than one chromosome pair. The lymph organ behavior is controlled by all main chromosomes in *tu* A_2, mainly by the second pair in *tu* B_3; in *melanotic e* 144 this character is very weakly controlled. The frequency of lamellocytes is controlled by the first and second pairs in *tu* A_2 and *tu* B_3, while in *melanotic e* 144 the second pair is the most responsible. Up to this point we have detected two multichromosomal systems of genes, also termed polygenes. The *tu*-genes have been further analyzed in two stocks—*tu* A_2 and *tu* B_3—with a sophisticated technique. Many

Table 8.—Effect of the main chromosome pairs on lymph organ behavior and on lamellocyte frequency in different tumorous stocks (*13*)

Stock	Chromosome*		
	1	2	3
	Lymph organ dissolved or disintegrated into lobes		
tu A_2	+	+	·
tu B_3	·	+	·
melanotic e 144	·	·	·
	Frequency of lamellocytes		
tu A_2	+	+	—
tu B_3	+	+	—
melanotic e 144	—	+	·
	Tumors		
tu A_2	—	+	—
tu B_3	—	+	—
melanotic e 144	—	+	—

*—: Replacing the chromosome is without effect; +: replacing the chromosome has an effect; ·: replacing the chromosome has a weak effect.

isogenic recombinant stocks which were produced carried different portions of the original second chromosome and a complementary section of the homologue, marked by *black* (45.5), *cinnabar* (57.5), and *vestigial* (67.0). Obviously, stock *b vg cn* is tumor free.

The results (table 9) show that *tu* A_2 has its *tu* factor(s) at the left of *b*, while the *tu* B_3's map at the right end, after *vg A*. The final step consisted in determining whether the *tu*'s in each stock are a simple factor or a complex of genes. To this purpose, several lines of *tu* B_3 were obtained, with the following genotype (table 10): *cn* (*cinnabar*), *c* (*curved*), *px* (*plexus*), and *tu* (*tumor*). The expectation was that, if *tu* were one single gene, all crosses between different lines would have to show tumors; if not, the factor must be a complex one. The results point clearly to a complex factor, since all F_1 offspring were tumor free. To summarize, for *tu* B_3 stock there is clear evidence that several *tu* genes map between 67 and the right chromosome end. There is no indication for a more precise localization of the *tu*'s. In other words, we do not know whether they compose one single sequence or whether they are irregularly scattered. In this connection one must remember that the chromosome is structurally normal. Similar evidence exists also with regard to *tu* A_2.

The complexity of the *tu* genotype cannot be generalized. Examples exist (*14*, *15*) of stocks where the *tu* factor is in one single locus. The nomenclature generally used labels *tu* the more or less mappable genes principally on the second chromosome. By this is meant that their action consists of control of melanin synthesis (*16*). The evidence in favor of this view is not very strong. There are also grounds for assuming that the complex and multichromosomal genotype, which controls the lymph organ behavior and the lamellocytes, also governs melanin synthesis, the so-called *tu* genes being responsible for switching on a stimulus capable of evoking melanization as a response. This will be discussed later.

TABLE 9.—Mapping *tu* genes in the second chromosome of stocks *tu* A_2 and *tu* B_3 (*17*)

Chromosome section		Crossing-over frequency	Map distance
tu A_2	*tu-b*	19. 4	20. 00 ± 3. 94
	tu-cn	28. 15	31. 75 ± 4. 58
	tu-vg	43. 7	67. 00
	b-cn	15. 5	16. 00 ± 3. 61
	cn-vg	15. 5	16. 00 ± 3. 61
tu B_3	*tu-b*	41. 9	59. 75
	tu-cn	28. 3	32. 00 ± 4. 94
	tu-vg	17. 2	17. 75 ± 4. 05
	b-cn	15. 7	16. 00 ± 3. 88
	cn-vg	11. 3	11. 50 ± 3. 38

TABLE 10.—Tumor frequency in crosses between identical genotypes *cn, c, px,* and *tu* B_3 (*13*)

Parental lines (%)		F_1	Number of individuals
♂	♀		
8. 6	63. 3	0. 0	102
63. 3	11. 1	0. 0	78
9. 3	4. 8	0. 0	96
9. 3	2. 6	0. 0	94
9. 3	8. 6	0. 0	252
8. 6	4. 8	0. 0	41

Irregularities in Hereditary Transmission

The genetic control of tumor production is exceptionally complex and sometimes reveals phenomena which are difficult to interpret according to the mendelian schemes. Two facts particularly merit mention:

1) The behavior of isogenic lines, kept through rigorous brother × sister mating for 4 or 5 generations (table 11—23 isogenic lines of *tu* B_3 stock), exactly parallels that in wild stocks (table 2). Since, in this case, genetic diversity within each line is eliminated, the only possible explanation is that "something" in the genotype has mysteriously changed. Since environmental changes might be involved, it should be added that environmental control was rigorous, especially as to temperature, and many countings of different lines were made simultaneously with the use of the same food sample.

2) The wild stock *Chieti-vermilion* was tested for tumors (scoring isogenic lines) in 1958 and 1959. A very low frequency was found, never exceeding 7%. A third test was made 6 months later in 9 lines, isogenic for the second and the third chromosome; 7 gave frequencies between 17.9 and 76.2%. From them were derived several sublines which were predominantly heterogeneous [table 12 (*13*)]. Contamination must be excluded, owing to the presence of *vermilion* as marker. In 1960 a new

TABLE 11.—Variation in tumor frequency in isogenic lines of *tu* B_3 (*13*)

Stable lines		8
Irregular lines	(oscillations>10%)	3
Gradually increasing	(increment>10%)	2
Gradually decreasing	(decrement>10%)	10
		23

TABLE 12.—Variation of tumor frequency in sublines from isogenic genotypes of *tu* B_3 (*13*)

Repetition of same chromosome	Culture of same repetition	(%)	Number of individuals
a	1	5. 5	72
	2	24. 6	61
b	1	60. 0	35
	2	77. 5	49
c	1	3. 3	30
	2	0. 0	35
	3	4. 2	24

group of isogenic lines was derived from the stock and examined through 7 generations obtained by brother × sister matings. A weak tendency toward a reduced tumor frequency was found, but the impression was that of a general stabilization; on the average, frequencies were of the order of magnitude of 1–7%.

Selection probably is unimportant as an explanation of such changes in a pedigree based on strict consanguinity. A phenomenon which might hypothetically be invoked is the existence of something similar to the controlling elements, susceptible to sudden localization change and influencing the action of the genes. Different manifestations of *tu*-genes could be the effect of presence or absence of a controlling element in their vicinity. The changing action of the hypothetical controlling element of *tu*-genes should be supposed to occur much more frequently than in maize; perhaps the whole phenomenon more closely resembles that described in *Salmonella* by Dawson and Smith-Keary (*18*).

Whatever the mechanisms involved in the irregularities referred to, the result is an enormous increase in genetic variability among tumorous flies.

Extrachromosomal Transmission

An extrachromosomal component of tumor transmission is indicated in the results of reciprocal crosses between stocks. These differences must be attributed to cytoplasm. More accurate data have been collected by transferring the chromosomes of a tumorous stock into cytoplasm of a tumor-free one or vice versa. Data presented in tables 13 and 14 (*17*) again demonstrate the presence of some cytoplasmic entity. The genotypes used for obtaining both combinations between chromosomes and cytoplasm have been constructed with all major chromosomes replaced (*i.e.*, except for the fourth pair) with marked *tu*-free chromosomes. Table 13 shows that a statistically significant difference exists between the combination where chromosomes and cytoplasm have the same origin and the one where a foreign and tumor-free cytoplasm is present. Table 14 shows that both stocks examined carry a small amount of tumors, even when the chromosomes are derived from stock without tumor.

This cytoplasmic effect does not cover the complexity of the extrachromosomal phenomena in tumorous stocks, because there is evidence that some entities are transmitted with equal efficiency through both gametes and are therefore more likely to be localized within the nucleus or very close to it.

The technique of obtaining tumorous flies from *tu*-free oocytes grafted (with the whole ovary) into tumorous individuals made possible the elimination of every contribution of the recipient chromosomes in the manifestation of the character. Unfortunately, a low proportion of tumors was also in the *Chieti-vermilion* donor stock (2.6 ± 0.174); hence only a statistical comparison could be made. However, grafting *tu* A_2 gave a percentage of 3.8 ± 0.26, and *tu* B_3 of 6.7 ± 0.24. Moreover, the descendants

TABLE 13.—Comparison between genotypes combined with homologous cytoplasm and heterologous cytoplasm (*17*)

	Males	Females	Total individuals counted
With own cytoplasm	55. 4%	77. 8%	265
Without own cytoplasm	23. 5%	31. 2%	212
Difference	21. 9	46. 6	
χ^2 (1 df)	27. 64	39. 45	

TABLE 14.—Tumor frequency in two stocks where the chromosomes are *tu*-less and the cytoplasm derived from tumorous stocks *tu* A_2 and *tu* B_3 (*17*)

Line	With tumors (%)		Without tumors
1	7 (3. 12)	Stock A_2 Total 224	217
2	5 (4. 34)	Total 115	110
1	3 (2. 40)	Stock B_3 Total 125	122
2	13 (9. 09)	Total 143	130
3	7 (5. 78)	Total 121	114

from grafts into *tu* A_2 revealed some thoracic tumors typical of *tu* A_2 and lacking in *tu* B_3. The phenomenon was repeatedly observed in several generations equally transmitted through both sexes. These results clearly indicate (*19*) that an extrachromosomal entity exists.

Another technique gave identical results—that of injecting larvae of tumor-free stocks with cell-free extract of tumorous flies. This technique also produced a proportion of tumors in the lines derived from the injected flies, and a few thoracic ones only, after injection with *tu* A_2 extract [table 15, in which data from 5 generations are pooled from Barigozzi (*20*)]. Both techniques have also been used with *Frd*. But, while ovary grafting failed to give a positive result (unpublished data) and the injections gave a very low percentage of transmitted pseudotumors, chromosome replacements demonstrated that an entity existed that was not linked to any chromosome, exhibiting a weakened *Frd* phenotype, which is also transmitted through both gametes, thus not matriclinously (text-fig. 1) (*15*).

These data on extrachromosomal transmission show that these mechanisms are very weak, as compared with those relating to the chromosomal genes. Moreover, these demonstrations are based on statistically

TABLE 15.—Transmission of pseudotumors in the offspring from genotypically tumor-free larvae injected with extract from tumorous larvae*

	Injected females	
	Transmission through females	Transmission through males
Total individuals	13, 782	17, 034
Total tumors	143	243
Thoracic tumors	32	31
Tumors (%)	1. 04	1. 43
Thoracic tumors (%)	0. 23	0. 18
$\frac{\text{Thoracic tumors}}{\text{Tumors}}$ (%)	22. 38	12. 76
Number of chromosomes	99	110

*Controls (lines isogenic for the second chromosome of *vermilion*)—number of chromosomes = 113; number of individuals = 5,236; number of tumorous individuals = 45; % tumorous = 0.86; thoracic tumors = 0.

Both parents $yw;\ \frac{CyL}{Pm}\ [Frd];\ \frac{Sb\ M\acute{e}}{H}$

Non[*Frd*]	[*Frd*]	% [*Frd*]	Cultures with [*Frd*]
2153	63	2.8	23/26

Control. Both parents $yw;\ \frac{CyL}{Pm};\ \frac{Sb\ M\acute{e}}{H};\ \frac{+}{+}$

Nontumors	Tumors (not of *Frd* type)	% *tu*	Cultures with *tu*
1149	3	0.3	3/20

TEXT-FIGURE 1.—Transmission of weak *Freckled.*

significant differences, and not on all-or-none effects. Nonetheless, it emerges that all tumorous stocks so far studied show the peculiar fact of having a dual transmission mechanism.

The unusual complexity of the hereditary units involved in controlling pseudotumors in *Drosophila* not only emphasizes the uniqueness of this phenomenon but also raises the problem of the relationship between chromosomal and extrachromosomal units. Clearly, one can conceive the two classes of units either as entirely unconnected (in the sense that the extrachromosomal units are simply added to the genes) or as connected by derivation (in the sense that the extrachromosomal units should be derived from the genes). The question has been posed (*13*, *15*, *16*) but remains unanswered. The possibility that the extrachromosomal state is the extrachromosomal phase of an episome (similar or not to a controlling element) has also been considered.

DISCUSSION

In an interpretation of the pseudotumors, based on genetic data involving only the blood cells, three facts emerge as undeniable: 1) The genotype of the tumorous flies is exceptionally complex. 2) The diversification of the tumorous genotypes is exceptionally high. 3) Individuals heterozygous for *tu*-genes are exceptionally frequent. From these starting points some hypothetical assumptions can be formulated. First, the heterozygous condition must be an advantage which we cannot yet define. Probably it involves the defense of the organism against external injuries. Consequently, stocks that lack *tu*-genes should have a lower fitness than those that have them. This seems to be only partially supported by the investigations of Di Pasquale and Koref (*8*). Therefore, we cannot be sure why genotypes controlling the production of a certain amount of tumors are so frequent. Whatever may be the significance of tumors being carried or not, there is an important problem concerning the action of the so-called *tu*-genes. For clarification, remember that we know two cases: 1) stocks not carrying inherited melanotic tumors but able to produce them after induction and 2) stocks that transmit them genetically.

The two mechanisms, induction and transmission, are not related to any difference as far as the phenotype is concerned. In induction, the stimulation to produce tumors is environmental and known. In transmission, it is internal and remains unknown. For a general explanation of the phenomenon, we reiterate that either: 1) *tu*-genes do really control the production of melanin, in which case the behavior of the lymph organ and of the lammelocytes are prerequisites for *tu* action; or 2) *tu*-genes produce the stimulus that triggers the action of the other genes, in which case they should control not only the release of lamellocytes but also their clumping and the production of melanin.

If we accept this second view, according to which the genes mapping in the second chromosome should not, properly speaking, be called *tu*, the origin of the inherited tumors should not be sought in parasites or viruses [the demonstration of which has been always illusory until now (*21–24*)]. Rather, we look within the organism for some form of trauma of unknown structure. Moreover, the induced melanizations would be in agreement with the fact that the *tu*-genes (located mostly on the second chromosome) are not necessary to produce melanin.

If we accept the first view, we must recognize that the etiology of the inherited tumors remains obscure, as does the link between induced and inherited melanotic tumors. The latter view, on the other hand, is more helpful on both points. It may, however, seem difficult to admit that a polygenic system might be responsible not only for lymph gland and lamellocyte behavior, but also for the production of melanin, which consists in the formation of polymeric molecules of indole. Such a production may be more easily ascribable to a major gene.

Whatever the final solution, the present data provide no evidence that the so-called melanotic tumors of *Drosophila* are neoplasms, if a neoplasm is characterized by uncontrolled cell division.

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Biochemical Basis of Hereditary Melanotic Tumors in *Drosophila*[1]

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SUMMARY

Appearance of melanotic tumors in the *tu(83.9)bw* strain of *Drosophila melanogaster* depends on the background genotype and the nutrition of the developing larvae. Only nutrition is open to specific, quantitative manipulation. Axenic nutritional studies show that deficiencies of dietary protein (casein), cholesterol, thiamine, pyridoxine, and magnesium ions raise tumor incidence among genetically homozygous flies. Excess dietary tryptophan or phenylalanine does likewise. Other deficiencies (or excesses), with equivalent effects on development, do not influence tumor penetrance so that the results must be specific to the effective treatments. These treatments are known to modify many unrelated metabolic pathways, and the problem is: How do they all influence the transformation of plasmatocytes into tumor cells? Feeding juvenile hormone-like substances raises, whereas the pupation hormone, ecdysone, lowers tumor frequency, and hence the transformation depends on this hormone balance. Since different dietary sterols also modulate tumor frequency, the cholesterol deficiency effect is probably through ecdysone, of which it is the precursor. Hormone secretion is also under nervous control, and the other effective deficiencies and excesses can modify the formation of neurohumoral substances. Specifically, inhibition or enhancement of the production of biogenic amines raises or lowers tumor frequency, as expected. An hypothesis is put forward which brings together the original, apparently unrelated, observations, and which may explain why so many different genes cause melanotic tumors.—Nat Cancer Inst Monogr 31: 291–301, 1969.

MELANOTIC TUMORS of *Drosophila* have two interesting characteristics that have not been much discussed. A large number of nonallelic mutant genes give the same phenotype or result in a phenotype conveniently put in the category *melanotic tumor*, as Harshbarger and Taylor (*1*) have noted. This is uncommon and is paralleled only by the 50 or so Minute mutants, which now seem to be deletions of t-DNA, scattered throughout the genome; *tu* genes are not deletions, but mendelizing recessives, generally confined to the second chromosome, although not exclusive to it. However, a limited survey of putative wild strains (*2*) showed that many

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[1] The financial support of the Agricultural Research Council is gratefully acknowledged.

carried subthreshold *tu* genes which could be exposed by some of the dietary treatments mentioned below, indicating that the *tu* genes so far studied represent only part of the *tu* gene population carried by *Drosophila:* the obvious, manifest "tip of the iceberg." So one has to ask why there are so many separate *tu* genes, for the number must be large, even if we cannot yet put a limit to it. This peculiarity may be relevant to the study of neoplasia.

Second, as already indicated, the penetrance of *tu* genes is very susceptible to environmental influences. The proportions of individuals in a population showing the tumors are affected by the genetic "background," by sex, physical agencies, and especially nutrition [references in (*1*) and (*3*)]. Generally speaking, genes of incomplete penetrance are readily influenced by environment, but the situation with respect to tumor genes is, at very least, an extreme example of this little studied phenomenon. And we have to ask why they are.

Of the various manipulations which will influence the proportion of tumorous individuals in a population, nutrition experiments are most likely to expose the metabolic lesion(s) causing the mutant phenotype. First, all the main metabolic pathways can be stressed individually by causing the organism to grow on diets deficient in particular nutrients; and second, these deficiencies can be graded, and the responses to them can be quantified and compared. This paper will examine the results of such a nutritional survey applied to only one tumor gene, *tu bw*, located at 83.9 on the second chromosome of *Drosophila melanogaster.* These results [2] confirm the general conclusion that *tu* gene penetrance is particularly susceptible to nutrition. However, the significant point is not just this, but that manipulation of most major metabolic pathways (even by this rather imprecise technique) has clear effects, positive or negative, on tumor incidence. The simple assumption that one defective enzyme (surely the product of the *tu* gene) causes the malfunctioning of a particular metabolic pathway is possibly correct, but clearly the interrelations of metabolism are so intimate that imbalances of other pathways may also modulate the functioning of the "mutant pathway." These interrelationships suggest why so many mutant genes may cause tumors, and also that our natural desire to ascribe single causes to the neoplastic state may be misplaced.

The technique of growing *Drosophila* larvae germfree on defined diets has already been described (*4*). It is only necessary to mention here that larvae need a protein source (casein), or the equivalent amino acids, choline (usually provided as lecithin), cholesterol, the B vitamins, and salts. Although larvae can synthesize nucleic acids, they grow more normally when provided with them (either as RNA or the equivalent nucleotides). Given a proper balance of these nutrients, larval growth is equivalent to that found when larvae are fed their usual diet of live yeast. Deficiencies of any of these essential nutrients necessarily slow growth, and the data below have been selected from treatments which have roughly equal effects on larval development rate so that equivalent states are compared. Excesses of the major nutrients also retard development, and these have also been studied. However, these excesses tell us very little about metabolism (*5*) except that slowing larval growth *per se* has little effect on tumor penetrance. Of the minor nutrients, only excess thiamine slows growth (*5*) and this by forming the well-known toxopyrimidine, which acts as an antipyridoxine when an excess of this vitamin is not provided. Other antivitamins also act like vitamin deficiencies. The effects of nutritional deficiencies are emphasized in this report.

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[2] I am grateful to Dr. Barrie Burnet, Dr. Peter A. Bond, Dr. Peter Bryant, and Mr. Jonathan Cooke for permission to quote unpublished data collected jointly with them.

Deficiencies of five growth factors in the diet caused the frequency of tumors to increase about 30% above a well-balanced control diet (table 1). Two pairs of these factors might be interrelated: the deficiencies of protein and pyridoxine, since pyridoxine is involved in protein metabolism; and deficiencies of thiamine and Mg^{++}, since Mg^{++} is often involved in the cofactor functions of thiamine, especially in carbohydrate metabolism. However, even with these possible interrelations each major area of metabolism (protein, carbohydrate, and fat) is affected and nothing is to be gained by comparing quantitative effects of deficiencies of such dissimilar nutrients. In fact, comparisons of the same *tu* gene in different genetic backgrounds show that the background genotype influences the response curves to any deficiency (*5*). Therefore the tumorous state is readily influenced by many metabolic disturbances.

Eight dietary deficiencies lowered tumor frequency in test populations (table 2). This was probably partially due to a slowing of growth, which raised the amounts of the effective substances listed in table 1, since excess cholesterol and excess casein lower the incidence of tumors (table 3), though they do not always do so when the *tu* gene is in other genetic backgrounds. On the other hand, excess RNA raises tumor frequency [due to the adenylic acid (*6*)] and it is difficult to predict what these contrary tendencies would do at the critical stage of larval growth [second to third instar ecdysis as demonstrated in (*7*)], since nothing is known about larval nutritional requirements at this early stage. The data (table 2) should therefore be taken at their face value, until we have reason to do the contrary.

TABLE 1.—Dietary deficiencies which raise tumor frequency—difference of percent tumor penetrance from control

Deficient supply	Tumor increase (%)*
Cholesterol	+25.4
Casein	+31.1
Thiamine	+15.5
Pyridoxine	+48.8
Mg^{++}	+31.8

*All increases significantly different from control value of 47.5%.

TABLE 2.—Dietary deficiencies which lower tumor penetrance—differences from control

Deficient supply	Tumor decrease (%)*
Lecithin	−19.8
Sucrose	−44.9
RNA	−44.6
Riboflavin	−39.8
Niacin	−18.3
Ca pantothenate	−30.7
Folic acid	−12.7

*Biotin deficiencies proved lethal to this strain; other *tu bw* strains show significant lowering of tumor penetrance at the same deficiency level (*4*). All decreases significant at the 1% level.

TABLE 3.—Effects of excesses of major nutrients—differences from control level

Excess supply	Tumor increase or decrease (%)
Cholesterol	−31.1
Lecithin	− 5.1
Sucrose	− 1.9
Casein	−43.8*
RNA	+41.5*

*Significantly different from control at 1% level.

Four deficiencies listed in table 2 may be tentatively grouped together (sucrose, riboflavin, niacin, and pantothenate), since they may all affect energy metabolism, though in different ways. Three others (RNA, folic acid, and biotin) may also influence a single process, since the two vitamins are necessarily involved in *de novo* synthesis of RNA by the larvae. That is, low folic acid or low biotin is equivalent to low RNA, and equivalent to a deficiency of nucleotides (*8*, *9*). Lecithin, the source of choline, is needed for the synthesis of phospholipid and therefore utilized either for the synthesis of cell structures or for energy (*10*).

While it is particularly interesting that nutritional deficiencies inhibit tumorigenesis in this system, the additonal possibilities this finding introduces to an already complex situation are rather daunting. Two further facts must be accounted for, and these are perhaps even more disheartening.

Plaine and Glass (*11*) confirmed earlier work showing that tryptophan and phenylalanine were remarkably tumorigenic when fed in excess to larvae. By feeding similar excesses (40mM amounts) of all the L-amino acids in the germfree diet, we have found (*12*) that this result is not indirectly due to alteration of the live yeast medium used by earlier workers, but a direct consequence of the excess of these two amino acids (table 4). Excess lysine is also significantly tumorigenic, to a lesser extent, but no single amino acid excess lowers the frequency of tumors as extra casein does (*12*). The specificity of the tryptophan-phenylalanine effect is further remarkable, since the other aromatic amino acid, tyrosine, and D-tryptophan and D-phenylalanine are completely ineffective (table 4).

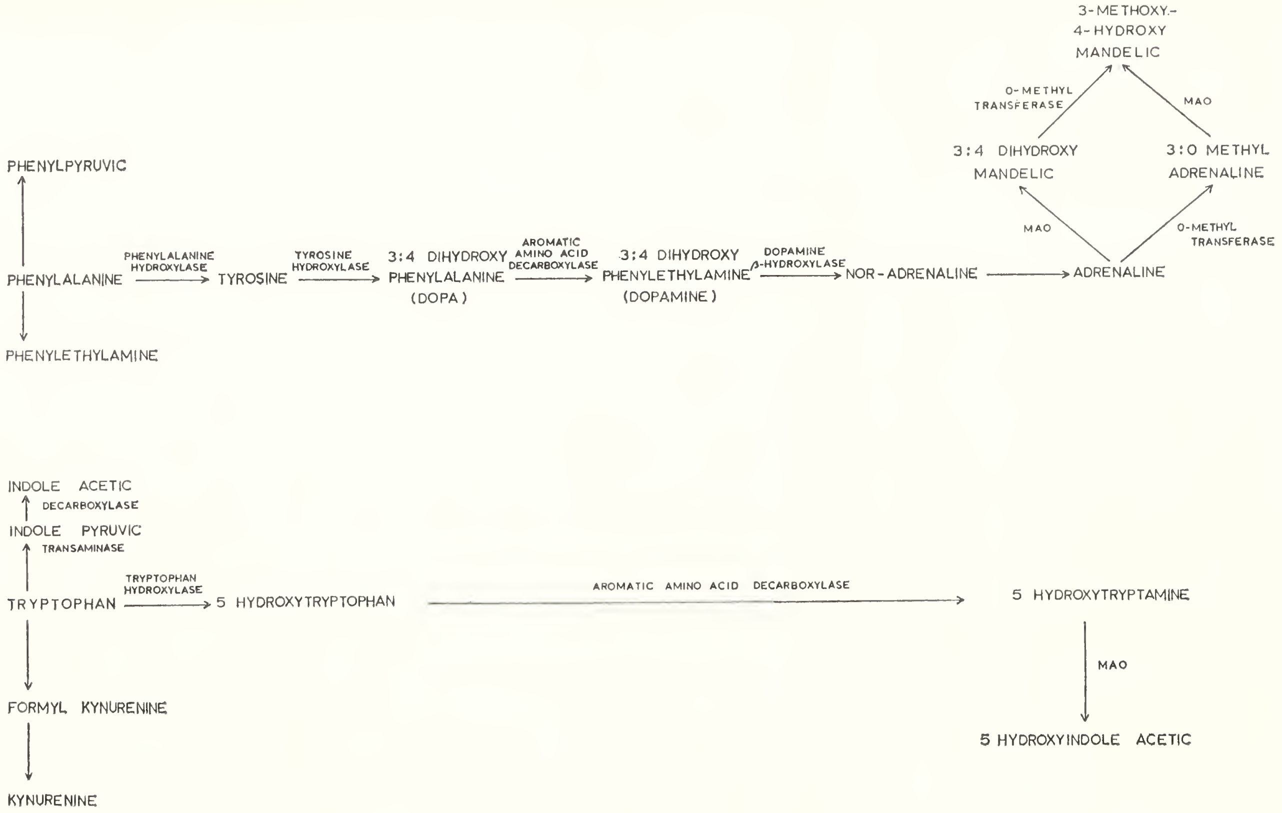
TABLE 4.—Effects of 40 mM additions of some amino acids to the control medium

	Tumor incidence (%)
Control	37. 2
+L-Arginine	42. 6
+L-Valine	30. 9
+L-Leucine	43. 0
+L-Isoleucine	33. 6
+L-Threonine	37. 7
+L-Phenylalanine	*75. 8
+L-Tryptophan	*85. 3
+L-Methionine	36. 4
+L-Lysine	*55. 9
+L-Histidine	44. 0
+L-Tyrosine	42. 8
D-Phenylalanine	46. 1
D-Tryptophan	27. 3

*Significant at the 1% level.

Because the phenylalanine and tyrosine effects are specific, whereas the other tumorigenic treatments alter metabolism more generally, Burnet and Sang (*12*) studied the consequences of these amino acids on larval metabolism. Their data show that neither amino acid leads to a reduction of the available amount of the tumorigenic nutrients listed in table 1. Second, analyses of the amino acid content of larval hemolymph (*12*) showed that both these amino acids were raised, as expected, when excess supplies were fed, and the balance of the other amino acids was thus altered. However, there was no common alteration of the amino acid pool which would indicate that both caused a single effective change, except that methionine and its sulfoxide were reduced in both cases. (Methionine was also shown to reduce the effects of phenylalanine and tryptophan when fed concurrently, but scarcely to an extent that might credibly account for the phenylalanine-tryptophan effects.) Third, a range of dissimilation products of these amino acids was tested to find out whether any was tumorigenic, and only two were found to be so: anthranilic acid and hydroxyanthranilic acid. Since these derivatives of tryptophan are unlikely dissimilation products in insects, probably they act independently, rather than by giving rise to a dissimiliation product common to phenylalanine and tryptophan. Subsequent compounds of the "aromatic pathway" (*13*) were not tumorigenic.

It is difficult to imagine that tryptophan or phenylalanine (or anthranilic acid) is directly tumorigenic, but this seemed to be the implication of Burnet and Sang's (*12*) data. However, two pathways had not been properly explored, those leading to the biogenic amines, noradrenalin and serotonin (text-fig. 1). The first was ignored for the good reason that noradrenalin is a derivative of tyrosine, and tyrosine is nontumorigenic. Serotonin, on the other hand, seemed unlikely to survive autoclaving and ingestion. However, both pathways could be approached indirectly *a*) by feeding a monoamine oxidase inhibitor (tranylcypromine), which would prevent the endogenous breakdown of adrenalin and serotonin, and *b*) by


3-METHOXY-
4-HYDROXY
MANDELIC
O-METHYL
TRANSFERASE
MAO
3:4 DIHYDROXY
MANDELIC
3:O METHYL
ADRENALINE
MAO
O-METHYL
TRANSFERASE
PHENYLPYRUVIC
PHENYLALANINE
PHENYLALANINE
HYDROXYLASE
TYROSINE
TYROSINE
HYDROXYLASE
3:4 DIHYDROXY
PHENYLALANINE
(DOPA)
AROMATIC
AMINO ACID
DECARBOXYLASE
3:4 DIHYDROXY
PHENYLETHYLAMINE
(DOPAMINE)
DOPAMINE
β-HYDROXYLASE
NOR-ADRENALINE
ADRENALINE
PHENYLETHYLAMINE
INDOLE ACETIC
DECARBOXYLASE
INDOLE PYRUVIC
TRANSAMINASE
TRYPTOPHAN
TRYPTOPHAN
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AROMATIC AMINO ACID DECARBOXYLASE
5 HYDROXYTRYPTAMINE
MAO
5 HYDROXYINDOLE ACETIC
FORMYL KYNURENINE
KYNURENINE

Text-figure 1.—The pathways involved in the synthesis of adrenalin and serotonin, and the steps affected by monoamine oxidases (MAO). The same enzyme carries out the decarboxylation step in both pathways, and pyridoxal-5-phosphate is the coenzyme.

feeding the drug reserpine, which depleted tissue amine stores (table 5). Both proved effective inhibitors of tumor formation, as also did lysergic acid diethylamide (LSD) (*12*), rather surprisingly. This result suggested that at least phenylalanine and tryptophan might act by interfering with either noradrenalin/adrenalin or serotonin synthesis, or both. Serotonin and adrenalin-like substances have been reported from the corpus cardiacum of insects (*14*), though their functions are unknown.

Since tranylcypromine was effective in preventing the breakdown of monoamines when fed in the diet, feeding noradrenalin and serotonin was tried, each in the presence of this monoamine oxidase inhibitor. The noradrenalin was rapidly oxidized by the feeding larvae, so that the medium became dark, and no response was obtained. On the other hand (table 6), serotonin is an effective inhibitor of tumor formation. Testing the effects of substances that block the uptake of adrenalin at α and β activity sites showed that these substances were effective, and also lowered the frequency of tumors (table 7). One might therefore expect that nor-

TABLE 5.—Tranylcypromine and reserpine effects on tumor frequency*

	Tumors (%)
Tranylcypromine (mg/100 ml)	
0	98
4	99
16	89
32	70
64	36
Reserpine (mg/100 ml)	
0	97
1	77
4	59
20	34

*In these and the following experiments, the control level of tumor penetrance was deliberately raised by lowering the *p*H of the culture medium.

TABLE 6.—Response to 5-hydroxytryptamine (serotonin) in the presence of monoamine oxidase inhibitor

Serotonin	0	0	40 mg/100 ml	80 mg/100 ml
Tranylcypromine	0	40 mg/100 ml	40 mg/100 ml	40 mg/100 ml
Percent tumors	95	72	52	41
Tumors per larva	1.54	1.41	0.76	0.55

TABLE 7.—Effects of feeding the α and β blocking agents, inderal and dibenyline, in the proportion 1:4

Amount of blocking agents (mg/100 ml)	0	100	200	400
Percent tumors	98	73	60	27
Tumors per fly	3.0	1.48	0.83	0.48

adrenalin/adrenalin would antagonize serotonin and raise the tumor incidence, but the technical difficulties of showing this have still to be resolved.

Unfortunately most of the substances in the tyrosine-adrenalin pathway cannot yet be successfully fed to larvae. However, because tyrosine itself is apparently not tumorigenic, this may prove to be an academic point, more particularly since it is well known (*15*) from studies of phenylketonuria that high levels of phenylalanine depress serotonin synthesis in organisms. It was therefore important to show that serotonin was effective when fed, not just during the whole of larval life, but immediately before the transformation of hemocytes to lamellocytes, at the end of the second larval instar. Transfer of 48-hour larvae from normal to serotonin-containing media gave a response very like that noted in table 6, showing that exogenous serotonin lowered tumor frequency when provided just before the cell transformation, which is the essential morphogenetic change in this particular tumor situation.

Since there is no *a priori* reason and no suggestive experimental data other than those just noted for thinking that these neurohumoral substances are themselves morphogenetic agents, the next question is: What do these biogenic amines influence? Rizki (*16*) suggested an answer to this question: the balance between juvenile hormone and the pupation hormone, ecdysone. This proposal has never been directly tested, but when juvenile hormone-mimicking substances (farnesyl methyl ether, etc.) and synthetic ecdysone became available, this could be done. Farnesyl methyl ether (table 8) dramatically raises tumor incidence when fed to larvae, and many other related substances behave similarly (*17*). Ecdysone, on the other hand, has the expected antagonistic effect, and lowers tumor incidence (table 8). It thus seems probable that Rizki's view is correct, but that excess juvenile hormone (or, more probably, a deficiency of ecdysone) at the time of the second larval ecdysis causes the cell transformation[3] which we identify as the melanotic tumor phenotype. Until hemocytes can be cultured and the transformation performed *in vitro*, it will remain difficult to prove this point categorically, since the situation is obviously more complex than the naive ecdysone/juvenile hormonal balance theory implies. For instance, Bryant (*18*) was unable to find any differences of development rates between tumor and nontumor strains, though this might be predicted from a hormone imbalance in the former strain.

Even if a hormone imbalance is accepted as the direct cause of the morphogenetic change leading to cell aggregation and tumor formation, the melanization being a secondary event occurring just before pupation, we have still to explain how so many different factors can influence this change, and why some raise, and others lower, tumor incidence. Because

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[3] Larvae respond to ecdysone fed at 48 hours when they weigh about 0.3 mg, and it is unlikely that they take in their own weight of food during the next 10 hours, by which time the cell transformation is certainly completed. That is, the effective ecdysone dose is about 0.03 μg. Similar calculations show that juvenile hormone substances are also effective at "humoral levels."

TABLE 8.—The influence of hormones on tumor frequency

Farnesyl methyl ether mM	0	0.5	1	2
Tumors per fly	1.6	1.85	2.1	2.8
Ecdysone μg/ml	0	5	10	20
Tumors per fly	0.82	0.86	0.54	0.27

the situation is complex, any explanation must be complex, too, and hence difficult to prove. The following outline hypothesis is tentative, given primarily as a basis for further experimentation.

The above data suggest that at, or immediately after, the second larval ecdysis, too little ecdysone (or too much juvenile hormone) is secreted and that this balance is regulated in some way by either noradrenalin or, more probably, by the serotonin level then present. It is not obvious how these neurohumoral substances act, but the data suggest that we are concerned mainly with the serotonin levels at the critical developmental stage. Any circumstance lowering either ecdysone synthesis or ecdysone release (*i.e.*, low serotonin levels) will increase tumor frequency in a population, and vice versa. The first tumorigenic deficiency (table 1) clearly is in this category, since an inadequate supply of cholesterol implies an insufficient synthesis of ecdysone, since it is synthesized from cholesterol (*19*). In support of this hypothesis, Cooke (*20*) has shown that different dietary sterols, in amounts sufficient for normal growth, lead to different levels of tumor incidence. However, it has not yet been possible to show categorically that sterols like 7-dehydrocholesterol which reduce tumor frequency are more readily synthesized to ecdysone than dihydrocholesterol which gives high tumor frequencies.

The effects of low pyridoxine (and low casein) may be attributed to their roles in the synthesis of serotonin, since the former is the cofactor for the aromatic amino acid decarboxylase that transforms 5-hydroxytryptophan to 5-hydroxytryptamine (serotonin). Thiamine (and Mg^{++}) are both essential for nerve action, which may imply that they are involved in serotonin release. High phenylalanine lowers serotonin synthesis, by some mechanism not yet understood (*21*), and the literature suggests that high tryptophan (or possibly high 5-hydroxytryptophan) acts similarly (*15*). We are currently examining these possibilities and the alternative, also suggested in the literature (*15*), that phenylalanine and tryptophan compete with serotonin in its uptake (*e.g.*, by rat brain), though the fact that tyrosine does not do so is against this alternative. Regardless of the outcome, it seems clear that the neurohumoral control mechanism, which certainly regulates ecdysone secretion in the well-known circadian rhythm pattern (*22*), must contribute a large number of the particular steps to the process leading to tumor formation.

Table 2 shows that many factors contribute similarly to lowering tumor frequency. All tumor-lowering dietary deficiencies are, in one way or another, directly involved in energy metabolism, and the standard inhibitors of this pathway, like fluoride, 2-4-dinitrophenol, or amytal, decrease tumor frequency when fed (table 9). However, it is not yet clear if the action

TABLE 9.—Effects of some inhibitors of energy metabolism on tumor frequency

Agent		Difference from control (%)
Fluoroacetate	1.0 mM	−53
Potassium cyanide	0.2 mM	−17
Rotenone	0.4 mg/ml	−53
2-4-Dinitrophenol	1.2 mM	−55
Diethylstilbestrol	0.04%	−63

of respiratory inhibitors (or dietary deficiencies) is directly on the hemocytes, where they might be expected to prevent the energy-requiring cell transformation, or if they act in some other fashion.

In this context, the response to thiamine might be taken as anomalous, since thiamine is certainly also involed in energy metabolism. However, a careful study of responses to thiamine shows that at a critical dose this vitamin also lowers the incidence of tumors, whereas less than this amount causes tumor frequency to increase. That is, the response to thiamine is not uniform. This raises one of the difficulties of studying this system, which we have so far ignored: Nutritional technique is a somewhat blunt instrument for the analysis of such a complex system, and any conclusions must, as yet, be subject to this implied reservation.

The purpose of this paper is not to "explain away" melanotic tumors in *Drosophila*, but to bring current data together in the framework of a hypothesis which shows not just the complexity of the system, but also something of the biochemical basis of this complexity. Future results may quickly overturn the hypothesis, but that is its purpose. The biochemical data, on the other hand, seem fairly firmly established, and they explain why many nonallelic genes may cause this particular tumor to appear, and why many environmental factors modulate tumor frequency. As in so many situations in developmental genetics, we are concerned with at least two metabolic pathways: one, complex in itself, regulating hormone balance, and the other influencing the ability of the target cells to respond to that balance. Many mutant genes and many environmental manipulations can modify either pathway and so cause, or inhibit, the appearance of tumors. What has still to be tested satisfactorily is whether environmental changes can themselves be tumorigenic in the absence of a genetic defect. Thus we are here concerned with the response system found in the presence of one particular mutant gene. Other "mutant systems" may be especially susceptible to other environmental manipulations (*23*).

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Tumors, Hormones, and Viruses in *Drosophila* [1]

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SUMMARY

1) The Bryan strain of Rous sarcoma virus (RSV) increases the incidence of tumors in larvae and pupae of Oregon R and multipurpose stocks but not in the *tu 36a* strain which has a higher incidence of spontaneous tumors. Simian virus (SV40) increases the incidence of tumors in the multipurpose stock. 2) Frequently the Bryan strain greatly increases lethal mutations on the X chromosome in *Drosophila.* In the concentrations used, neither the Schmidt-Ruppin strain nor strain 559 increases lethal mutations significantly. The number of chromosomal aberrations and nondisjunctions in both germ and somatic cells is increased by the Bryan strain and to a lesser extent by strain 559 and the Schmidt-Ruppin strain. Treating larvae of *Drosophila* beginning at 3 days of age yields more chromosomal abnormalities with the Bryan strain (1:10) than at 7 days of age. 3) More lethal mutations were found than expected in the heterochromatic region of the X chromosome when 29 were analyzed after treatment with the Bryan strain. 4) The Bryan, Schmidt-Ruppin, and RAV-1 strains of RSV and SV40 reduce the mean size of chromosomal puffs at selected loci on the X and third chromosomes. 5) The size of chromosomal puffs in salivary glands is increased after exposure to proteinic brain hormone, inokosterone, iso-inokosterone, and Ponasterone A. Crystalline ecdysones and chromosomal puffs each have characteristic stimulation and response. 6) Evidence suggests that both RNA and DNA viruses not only increase the number of tumors but also alter the DNA template and interfere with elaboration of messenger RNA in *Drosophila.*—Nat Cancer Inst Monogr 31: 303–321, 1969.

NUMEROUS STRAINS of *Drosophila* develop melanotic tumors spontaneously (*1*); most of these tumors consist of polygonal cells with fusiform stroma (fig. 1), and the relative roles of accretion and proliferation in

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[2] Several colleagues supplied a portion of the viruses and hormones used in these studies. Rous sarcoma virus was obtained through the courtesy of Dr. R. Holdenreid from the Program Resources and Logistics Section, National Cancer Institute, Bethesda, Md.; proteinic brain hormone was supplied by Dr. M. Kobayashi, 1121 Matsunoki, Suginami-Ku, Tokyo, Japan; and crystalline ecdysones were supplied by Professor K. Nakanishi, Department of Chemistry, Tohoku University, Sendai, Japan, and Professor T. Takemoto, Pharmaceutical Institute, Tohoku University.

their formation are not always clear. The similarity of the entire process to that of reaction to injury in insects and the confusion with normal hemocytes have been pointed out many times. We first noted that ecdysones from the ring gland inhibit their growth (*2, 3*), which explains the regression of these tumors in the larval stages and persistence into imago as pigmented detritus (fig. 2). Through appropriate experimental design, in any experiment reported we have always attempted to eliminate the possibility that physical trauma was responsible for a reaction simulating pigmented tumors.

In earlier work (*4*) it was found that these tumors appear with a characteristic range of frequency in isogenic strains. The penetrance varies from less than 1.0 to over 99.0% in various stocks, and for unknown reasons genes for susceptibility are more frequent on the second chromosome than elsewhere. Multiple genes are rarely allelomorphic when tested by interstrain crosses, but genetic control is usually a complex of main genes and auxiliary suppressors and/or enhancers scattered throughout the genome (*1*). Certain chemical compounds, such as 3-methylcholanthrene (*5*) and nitrogen mustard (*6*), can increase the mutation rate. Caloric restriction is associated with diminished penetrance (*7*), but specific alterations in dietary proteins, vitamins, and ions have raised the incidence of these tumors (*8*). Compounds with juvenile hormone activity increase the number of tumors, an expected action, since ecdysones inhibit the appearance of gross tumors (*2, 3*).

In the *tu 48a vg bu* strain, the presence of genoids for CO_2 sensitivity depressed (*9*) the numbers of tumors detected in many experiments we performed with 3 strains of virus. In a continuation of studies on the effect of viruses, *Drosophila* larvae were exposed to Rous sarcoma virus (RSV) and the number of tumors was increased in the strains tested (*10*). In addition, there were more mutations and chromosomal abnormalities than in control cultures. We have now extended these studies to include simian virus (SV40) as well as different strains of RSV and have tested for possible alterations in incidence of tumors, chromosomal aberrations, and mutations. The size of puffs in salivary chromosomes was also determined, since inhibition was suggested in earlier experiments.

The strains of *Drosophila* developing tumors thus constitute a situation as complex as mammalian systems responsible for unusual aggregations of cells. Therefore the reason for utilizing *Drosophila* resides chiefly in unique technical advantages, such as salivary chromosomes and genetic methodology, the use of this organism offers when compared to other metazoans. With this in mind, studies were carried out to determine effects not only on incidence of tumors, but also on mutations, chromosomal aberrations, and puffing pattern following exposure to oncogenic viruses and crystalline hormones. A summary of these results follows.

METHODS

In the first experiments (*10*) mortality was high among those larvae treated, and black pigmented tumors in both larvae and pupae were found adjacent to the gut. None of the individuals with these tumors survived. Most interesting was the increased rate of lethal mutation on the X chromosome when males were exposed to RSV in the medium. Also there were increased nondisjunction and losses of the Y chromosome. A few mosaics of the eye and a few visible mutations were encountered. Encouraged by these results, we repeated the experiment with the Bryan strain in 1:1 and 1:50 dilutions. Similar studies were carried out with the Schmidt-Ruppin, 559, and Rous-associated virus (RAV1) strains. Later, SV40 was used in an initial group of experiments with DNA viruses.

The tandem X•Y chromosome stock, with autosomal and allosomal markers reported to be so useful by Muller and Oster (*11*), was used in this experiment to detect lethal mutations, losses of the Y, nondisjunctions, translocations, F_1 sterility, incidence of tumors, mosaics, and gynandromorphs. Although some visible mutations have been analyzed and confirmed, they have been grouped with phenocopies under that heading. The Muller-5 X chromosome was used to determine rate of lethal mutations in the Oregon R strain. Twenty-nine lethal mutations obtained after exposure to the Bryan strain of RSV were localized in crossover studies. Environmental conditions were stabilized as much as possible for determination of tumor incidence.

Five index puffs were chosen for measurement in studies on response of salivary gland chromosomes to hormones and virus. These were located at 2B on chromosome I and 71, 72 and 74, 75 on the left arm of chromosome III. Salivary glands were removed after exposure of third-instar larvae to injected hormone for a specified interval. In the groups observed for possible effects of virus, the larvae were exposed to concentrations of virus (described below) added to the medium, and salivary glands were removed when they had reached the third instar. Data on size of puffs in aceto-orcein preparations are expressed as ratio of diameter of puff to adjacent band in order to minimize inequalities in pressure at the time chromosomes were compressed.

The initial concentration of RSV was 1 g equivalent per milliliter suspended in 0.4 ml of modified Ringer's solution containing no calcium or magnesium. It was added to culture medium so that it constituted one-tenth the final concentration. The SV40 cultures contained 4×10^6 plaque-forming units per milliliter. Control cultures contained either saline alone or virus inactivated by irradiation. Viability of RSV was tested by inoculation into chicks and observing them for the appearance of sarcomas. The plaque-assay method was used for assessing potency of SV40.

Our early work indicated that ecdysones constitute a group of hormones (*12*), and ecdysones other than α-ecdysone and ecdysterone, originally extracted and purified by Butenandt, Karlson, Huber, Hoffmeister, and their colleagues, are now known (*13–16*). We have used three ecdysones and have tested their individual action on pattern of puffing in salivary gland chromosomes. The sources for extracting ecdysones now include plants such as fir and bracken, and the structural formulas have been determined for several of them (*15*, *17–20*). Three of these crystalline hormones were obtained, and their action on salivary gland chromosomes was tested. In addition, proteinic brain hormone supplied by Dr. Kobayashi has been used in similar studies (*21*).

RESULTS

Effect of Oncogenic Viruses

Lethal Mutations and Chromosomal Abnormalities

In table 1 are given the results of 6 experiments in which the rate of visible and lethal mutations, the appearance of mosaics, and the number of chromosomal abnormalities in *Drosophila* treated with the Bryan strain of RSV were studied. The most striking finding was the increased rate of lethal mutations following exposure to the Bryan virus: 2.11% for the 1:50 dilution and 1.73% for the 1:1 dilution, in contrast to 0% in control cultures. Nondisjunction and losses of the Y were clearly greater in the cultures treated with virus than in the control cultures, although nondisjunction was also found in the control cultures. The presence of gynandromorphs and mosaics indicates that the chromosomal damage discovered in the germ cells also was in progress in the somatic cells. Translocations in control and treated cultures were few; only 5 were detected during the 6 experiments. Many F_1 progeny were sterile, and the percentage of sterility was higher in those from parents treated with RSV.

When the experiment was repeated (table 2) with the Schmidt-Ruppin strain in dilutions of 1:1 and 1:50, the number of lethal mutations was small. In a subsequent study (tables 3 and 4), in which the age at the time of treatment varied and the dilution of the Schmidt-Ruppin strain was 1:10, more lethal mutations were found. No translocations were scored.

TABLE 1.—Mutations, mosaics, and chromosomal abnormalities in *Drosophila* treated with Rous sarcoma virus (Bryan strain)

Aberration	1:1 Dilution		1:50 Dilution		Control	
	Number/ Total	Percent	Number/ Total	Percent	Number/ Total	Percent
Loss of Y	4/3,055	0. 13	33/11,214	0. 29	1/3,396	0. 03
Nondisjunction	24/7,621	0. 31	130/26,561	0. 48	15/8,808	0. 17
Gynandromorphs	5/7,621	0. 07	7/26,561	0. 03	0/8,808	0. 00
Mosaics	56/7,621	0. 73	65/26,561	0. 24	9/8,808	0. 10
Phenocopies	8/7,621	0. 10	16/26,561	0. 06	0/8,808	0. 00
Lethal mutations	18/1,041	1. 73	83/3,941	2. 11	0/1,412	0. 00
Translocations	0/559	0. 00	5/2,668	0. 18	0/865	0. 00
F_1 sterility	56/238	23. 53	143/1,057	13. 53	26/394	6. 60

TABLE 2.—Mutations, mosaics, and chromosomal abnormalities in *Drosophila* treated with Rous sarcoma virus (Schmidt-Ruppin strain)

Aberration	1:1 Dilution		1:50 Dilution		Control	
	Number/ Total	Percent	Number/ Total	Percent	Number/ Total	Percent
Loss of Y	1/1,025	0. 10	4/5,889	0. 07	0/2,466	0. 00
Nondisjunction	9/2,412	0. 37	102/13,995	0. 72	12/5,952	0. 20
Gynandromorphs	1/2,412	0. 04	0/13,995	0. 00	0/5,952	0. 00
Mosaics	17/2,412	0. 70	99/13,995	0. 71	21/5,952	0. 35
Phenocopies	1/2,412	0. 04	1/13,995	0. 01	0/5,952	0. 00
Lethal mutations	0/238	0. 00	3/1,088	0. 29	0/481	0. 00
Translocations	0/229	0. 00	0/886	0. 00	0/422	0. 00
F_1 sterility	18/245	7. 35	98/984	9. 96	30/482	6. 23

Nondisjunction and mosaics were increased, particularly in the 1:50 dilution, suggesting that the Schmidt-Ruppin virus does have some effect, but not as striking an effect as with the Bryan strain.

Generally, the Bryan strain had a greater effect on mutations, mosaics, and chromosomal abnormalities when it was added to cultures at 3 days of age (table 3) than when added at 7 days of age (table 4). However, this test was not nearly as large as the one previously reported (table 1) for the Bryan strain. F_1 sterility was increased to about the same degree when the Bryan strain was added to cultures either at 3 or 7 days; sterility was not increased appreciably over controls when Schmidt-Ruppin and 559 strains were used. Again, the Schmidt-Ruppin strain increased nondisjunction, mosaics, and losses of the Y, but the number of lethal mutations was not impressive. Exposure for the two periods of time did not yield very pronounced differences. Although lethal mutations were found following treatment with strain 559, these rates were not much different from control rates. The number of mosaics and the amount of nondisjunction were increased with the strains of RSV, but there was no trend for those cultures exposed longer toward a greater number of mosaics and nondisjunctions. When an additional test was done with the Bryan strain of virus and Oregon R strain of *Drosophila* (table 5), the lethal mutation rate was increased in a group treated with virus in dilution of 1:50.

Crossover analysis (table 6) of lethal mutations, which appeared on the X chromosome after treatment of the multipurpose and Oregon R strain with Bryan high-titer RSV, yielded an interesting pattern of localization. Lethal mutations found after exposure to this RNA virus were distributed in 15 of the cases along the length of the X chromosome, but 14 of them were on the right end in or near the heterochromatin.

Incidence of Tumors

When the incidence of tumors in 3 different strains of *Drosophila* treated with the Bryan strain of RSV was determined (table 7), the multipurpose and the Oregon R stocks were found to have more tumors (figs.

Table 3.—Mutations, mosaics, and chromosomal abnormalities in *Drosophila*, 3 days of age, treated with Rous sarcoma virus (Bryan, Schmidt-Ruppin, and 559 strains)

Aberrations	Bryan		Schmidt-Ruppin		559		Control	
	Number/Total	Percent	Number/Total	Percent	Number/Total	Percent	Number/Total	Percent
Loss of Y	2/851	0. 24	6/2, 610	0. 23	2/2, 184	0. 09	0/339	0. 00
Nondisjunction	7/1, 896	0. 37	21/5, 786	0. 36	6/4, 819	0. 12	1/761	0. 13
Mosaics	14/1, 896	0. 74	48/5, 786	0. 83	24/4, 819	2. 52	2/761	0. 26
Phenocopies	2/1, 896	0. 11	1/5, 786	0. 02	1/4, 819	0. 02	0/761	0. 00
Lethal mutations	4/184	2. 13	5/590	0. 85	3/536	0. 56	0/185	0. 00
Translocations	0/125	0. 00	0/381	0. 00	0/347	0. 00	0/141	0. 00
F_1 sterility	32/207	15. 46	54/630	8. 57	43/549	7. 83	10/195	5. 13

Table 4.—Mutations, mosaics, and chromosomal abnormalities in *Drosophila*, 7 days of age, treated with Rous sarcoma virus (Bryan, Schmidt Ruppin, and 559 strains)

Aberrations	Bryan		Schmidt-Ruppin		559		Control	
	Number/Total	Percent	Number/Total	Percent	Number/Total	Percent	Number/Total	Percent-
Loss of Y	1/1, 638	0. 06	0/2, 281	0. 00	0/1, 908	0. 00	0/1, 299	0. 00
Nondisjunction	13/3, 793	0. 34	20/5, 038	0. 40	12/4, 376	0. 27	4/2, 943	0. 14
Mosaics	6/3, 793	0. 16	38/5, 038	0. 75	6/4, 376	0. 14	0/2, 943	0. 00
Phenocopies	1/3, 793	0. 03	0/5, 038	0. 00	0/4, 376	0. 00	0/2, 943	0. 00
Lethal mutations	1/694	0. 14	1/740	0. 14	3/553	0. 54	0/327	0. 00
Translocations	0/503	0. 00	0/476	0. 00	0/354	0. 00	0/178	0. 00
F_1 sterility	134/828	16. 18	67/785	8. 54	40/623	6. 42	23/350	6. 57

3 and 4) than control cultures at both the 1:1 and 1:50 dilutions, whereas the *tu 36a* strain that develops tumors spontaneously did not have any increase in tumors. The mortality rate was quite high in the multipurpose stock after treatment with Bryan strain. Although no systematic study had been carried out previously on the multipurpose stock for the presence of tumors, apparently tumors do appear in this strain without any treatment and the virus may increase their number. The same is true for the Oregon R stock in these experiments and an additional study which included more observations (table 5). Virus did not increase significantly the number of tumors that appear in the *tu 36a* strain, which develops more tumors spontaneously than either of the other two stocks.

TABLE 5.—Incidence of tumors and rate of mutations in Oregon R *Drosophila* after treatment with Bryan strain of Rous sarcoma virus

Class	1:1 Dilution		1:50 Dilution		Control	
	Number/ Total	Percent	Number/ Total	Percent	Number/ Total	Percent
Mutations						
Lethal	3/511	0. 59	13/582	2. 23	0/127	0. 0
Semilethal	4/511	0. 78	12/582	2. 06	0/127	0. 0
Phenocopies	38/5,913	0. 64	28/5,243	0. 53	2/906	0. 22
Tumors	79/5,913	1. 34	74/5,243	1. 41	0/906	0. 0

TABLE 6.—Distribution of virus-induced lethal mutations along the X chromosome

Loci	Number of lethal mutations (Oregon R)	Number of lethal mutations (multipurpose)	Total	Percent
0. 0–2. 5		5	5	17
2. 6–5. 0	1		1	3
5. 1–7. 5	2		2	7
7. 6–10. 0				
10. 1–12. 5				
12. 6–15. 0	1		1	3
15. 1–17. 5	1	3	4	14
17. 6–20. 0				
20. 1–22. 5				
22. 6–25. 0				
25. 1–27. 5				
27. 6–30. 0				
30–1–32. 5				
32. 6–35. 0				
35. 1–37. 5	1		1	3
37. 6–40. 0				
40. 1–42–5				
42. 6–45. 0	1		1	3
45. 1–47. 5				
47. 6–50. 0				
50. 1–52. 5				
52. 6–55. 0				
55. 1–	6	8	14	48
Total	13	16	29	

When effect of length of exposure to virus was tested (table 8), the number of tumors was higher than control values in the Bryan, Schmidt-Ruppin, and 559 strains. When the effect of the length of exposure of the multipurpose stock to each of 3 different strains of RSV was tested, tumor incidence (table 8) was higher in Bryan and Schmidt-Ruppin strains when exposure began at 3 days rather than at 7 days of age. The number was not increased in strain 559 after the longer exposure to virus. As before in a different concentration, the virus was very toxic.

Preliminary results (table 9) from a study with DNA viruses demonstrate an elevated incidence of tumors and a high mortality (24.2%) in the

Table 7.—Incidence of tumors in 3 strains of *Drosophila* after treatment with Bryan strain of Rous sarcoma virus (RSV)

Strain	Treatment	Tumors		Mortality		Total No. treated
		Number	Percent	Number	Percent	
Multipurpose	RSV, 1:1	24	4. 8	151	30. 1	502
	RSV, 1:50	126	8. 7	469	32. 3	1,451
	Control	2	0. 3	31	5. 4	572
Oregon R	RSV, 1:1	20	2. 6	18	2. 4	760
	RSV, 1:50	19	1. 4	2	0. 2	1,320
	Control	2	0. 3			640
tu 36a	RSV, 1:1	18	12. 5	10	6. 9	144
	RSV, 1:50	46	10. 6	1	0. 2	434
	Control	53	14. 0	1	0. 3	378

Table 8.—Pupal lethality and tumor induction in *Drosophila melanogaster* treated with Rous sarcoma virus (Bryan, Schmidt-Ruppin, and 559 strains), 1:10

Strain	Age at treatment (days)	Mortality (%)	Tumors (%)	Total No.
Bryan	7	25. 1	17. 8	287
	3	43. 4	35. 9	159
Total		31. 6	24. 2	446
Schmidt-Ruppin	7	18. 1	11. 2	376
	3	38. 5	20. 0	200
Total		25. 2	14. 2	576
559	7	17. 8	9. 6	314
	3	25. 3	9. 7	186
Total		20. 6	9. 6	500
Control-O	7	1. 7	1. 3	240
Control-Y	3	3. 5	2. 3	140
Total		2. 4	1. 7	380

multipurpose stock following exposure to SV40. Equivocal results were obtained when Oregon R larvae were scored. Also mortality was lower when the Oregon R strain was treated.

Size of Chromosomal Puffs

The results of treatment with the Bryan and Schmidt-Ruppin strains of RSV on puffing pattern in salivary gland chromosomes of Oregon R larvae appear in table 10 and text-figures 1a through 3b. The Bryan strain was used in a dilution of 1 : 10. The Schmidt-Ruppin strain was used in a dilution of 1 : 20, since it was too toxic diluted 1 : 10 for the two-day eggs of the Oregon R stock to develop after exposure. The greatest effect was on puff 2B, with the number of very large puffs reduced in a pronounced fashion. Also the mean value for the remaining puffs was reduced, but less extensively. The Bryan strain had a slightly greater effect than the Schmidt-Ruppin strain. There was a trend for higher concentrations of SV40 and RAV1 to inhibit puffing more effectively.

TABLE 9.—Incidence of tumors in *Drosophila* after treatment with simian virus (SV40)

Strain	Treatment	Tumors		Mortality		Total Number treated
		Number	Percent	Number	Percent	
Oregon R	SV40, 1:1	6	0. 7	7	0. 8	838
	Control					316
Multipurpose	SV40, 1:1	51	8. 9	139	24. 2	575
	Control	4	2. 2	5	2. 8	179

TABLE 10.—Tumor incidence following treatment of pre-imaginal stages with oncogenic virus

Stock	Virus administered*	Tumors (%)	Total No. observed	*P*
Multipurpose	Control	2. 3	2, 230	—
	BH-RSV, 1:1	5. 2	852	<0. 005
	SV40, 1:1	8. 5	1, 396	<0. 005
	RAV1, 1:1	4. 2	1, 702	<0. 005
	BH-RSV, 1:50	7. 9	1, 846	<0. 005
	SV40, 1:50	7. 8	742	<0. 005
	RAV1, 1:50	8. 1	1, 530	<0. 005
Oregon R	Control	0. 3	1, 320	—
	BH-RSV, 1:1†	2. 6	760	<0. 005
	SV40, 1:1†	0. 9	1, 337	0. 05–0. 10
	BH-RSV, 1:50†	3. 2	801	<0. 005
	SV40, 1:50†	1. 0	314	0. 25–0. 5

*BH-RSV = Bryan strain of Rous sarcoma virus; SV40 = simian virus; RAV1 = Rous-associated virus.
†Yates' correction applied.

Effect of Hormones

Pattern of Puffing

In order to facilitate comparison of response of individual puffs to respective hormones (ratio between puff and adjacent band), response is expressed as the percentage increase or decrease (preceded by minus sign) when compared to control preparations (table 11, text-figs. 3A and 3B). Puff 72 responded promptly to ecdysterone, but puffs without additional hormone reached the same size or greater at the end of 90 minutes. Response of puff III-71 was delayed until the 40-minute interval, both with inokosterone and with Ponasterone A. Puffs I-2B and III-72 were sustained more consistently when inokosterones were given than when Ponasterone A was given. Puff III-75 responded differently to inokosterone and iso-inokosterone (ecdysterone). When the concentration of proteinic brain hormone was increased, relatively greater response occurred after 20 minutes. Brain hormone clearly increased the amount of puffing at all loci (text-figs. 3A and 3B).

DISCUSSION

These data provide additional information about heritable melanotic tumors in *Drosophila* and present strong evidence that oncogenic viruses can increase the numbers appearing in a given strain. Differences both in response between strains and effectiveness between viral agents are also apparent from the results. Dosage effects are somewhat puzzling. Longer exposure yielded more tumors when a difference was present, but a higher concentration was often less effective than a lower concentration. Mortality was high in many cultures and no doubt affected results for incidence of tumors.

The increased number of lethal mutations in many experiments was very impressive.[3] Interestingly, the distribution of those localized was skewed, more appearing on the right end of the X chromosome than expected. Although transformation of mammalian cells by oncogenic virus has been reported (*22*, *23*), these studies are the first to show that mutations are induced by viruses. So far, experiments with fluorescent antibodies have failed to localize RSV antigen on salivary gland chromosomes. Increased percentages of mosaics and phenocopies indicate somatic damage by RSV in addition to the changes in germ cells. The number of tumors appearing in F_1 and F_2 generations was often greater than in control cultures, but no virus has been recovered so far to indicate viable passage.

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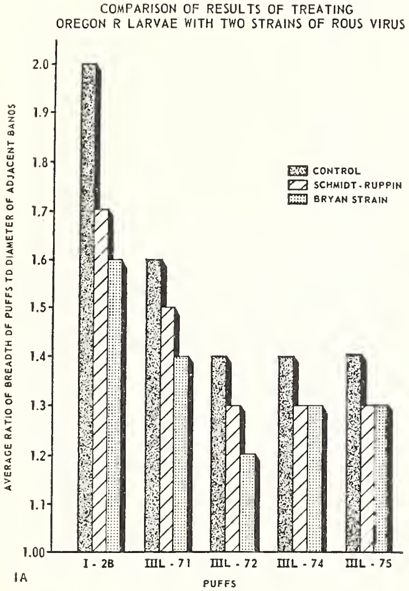
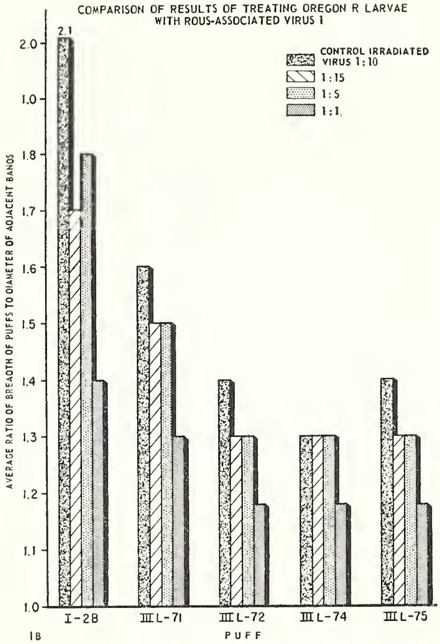
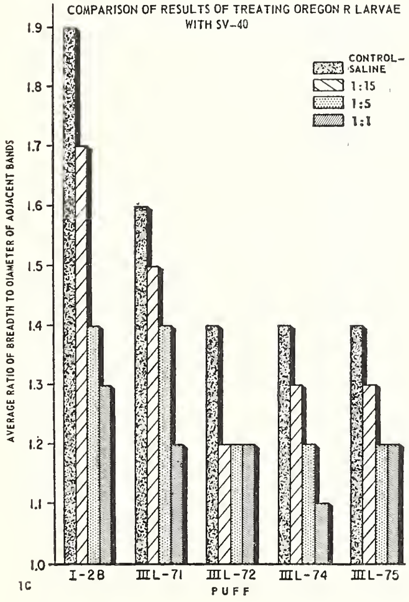
[3] The high rate of mutations has not been sustained in subsequent experiments featuring injection of Rous virus.



1A

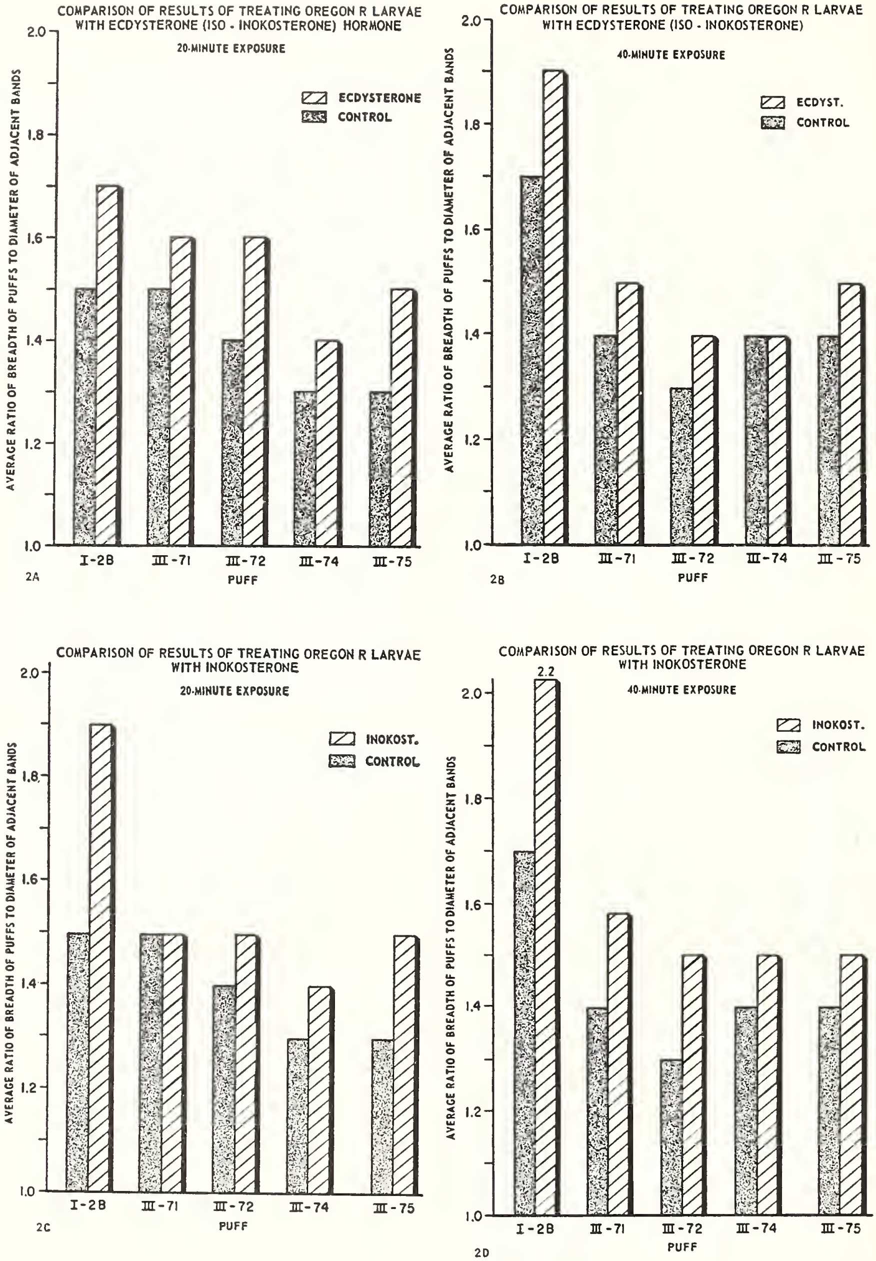


1B

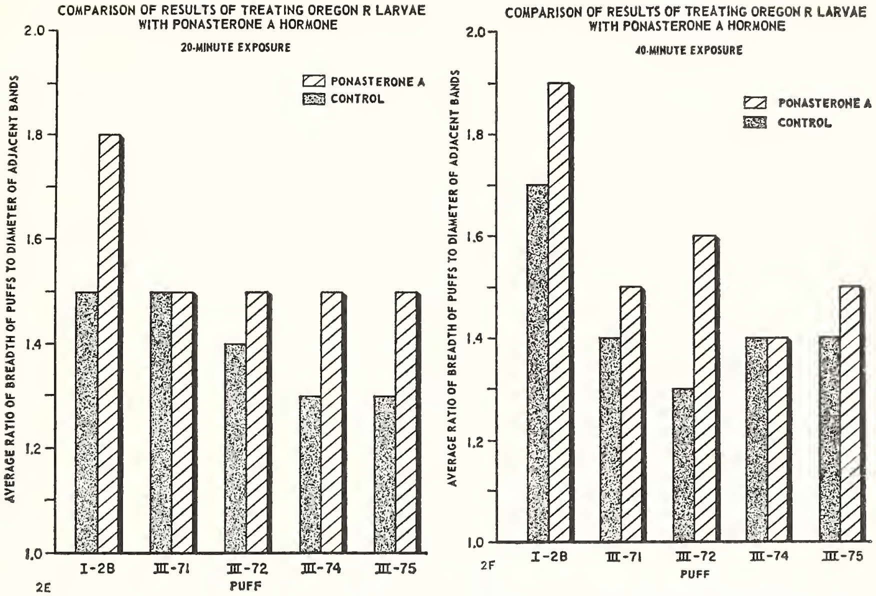


1C

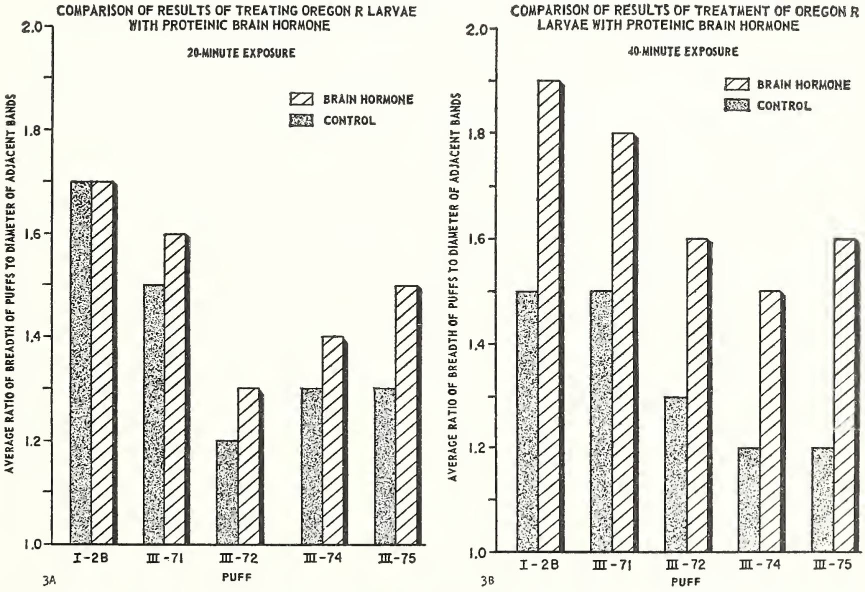
TEXT-FIGURES 1A, 1B, AND 1C.—Inhibition of puffing in salivary gland chromosomes by oncogenic viruses.



TEXT-FIGURES 2A, 2B, 2C, AND 2D.—Puffing pattern in salivary gland chromosomes after treatment with crystalline ecdysones—continued.



TEXT-FIGURES 2E AND 2F.—Puffing pattern in salivary gland chromosomes after treatment with crystalline ecdysones.



TEXT-FIGURES 3A AND 3B.—Puffing pattern in salivary gland chromosomes after treatment with proteinic brain hormone.

TABLE 11.—Percentage change in mean ratio of breadth of puffs to diameter of adjacent bands: Action of hormones on rate of puffing

Hormone	Minutes	Puff				
		2B	71	72	74	75
Ecdysterone (iso-inokosterone)	20	13	7	14	8	15
	40	12	7	8	0	7
	60	−5	0	23	−7	−12
	90	0	0	−6	0	−12
Inokosterone	20	27	0	7	8	15
	40	30	14	15	7	7
	60	−5	0	15	−7	−19
	90	6	−18	−12	−7	−12
Ponasterone A	20	20	0	7	15	15
	40	12	7	23	0	7
	60	0	−6	15	−7	0
	90	0	−18	−12	0	−6
Proteinic brain hormone*	20	0	7	8	8	15
	40	27	20	23	25	33
Proteinic brain hormone†	10	16	23	25	0	7
	20	18	13	17	23	38
	40	20	7	8	33	33

*One *Bombyx* unit per 100 μg.
†One *Bombyx* unit per 50 μg.

From one experiment to the next there has been considerable variation in the number of lethal mutations and chromosomal abnormalities encountered in comparison to those experiments in which virus was not given. Each time, however, the value for at least one of the parameters measured was elevated. The numbers of tumors found in all studies done so far are summarized in table 10; increased incidence was sustained after treatment in all cases except for the Oregon R strain treated with SV40.

Use of crystalline hormones confirms the action of ecdysones to increase puffing. In addition, each hormone stimulates and each locus studied responds somewhat characteristically. This suggests that the sequence and timing of genic derepression are partly controlled by independent concentration of different hormones in the mixture of ecdysones. This must be of considerable significance during metamorphosis. Suppression of puffing by exposure to oncogenic virus, which is demonstrated both for RNA and DNA viruses, suggestively localizes one type of action between derepression and elaboration of messenger. In addition, the template can be changed and chromosomal mechanics deranged by virus. Appropriate manipulation of genic, hormonal, and viral activity later may expose other, even more significant, details. How these effects relate to tumorigenesis is not clear. Inability to detect virus, either incorporated or as a more casual passenger, may be related as much to uncertain methodology as to actual transitory presence of the agent. Alteration in size of puffs can result from many circumstances and is not necessarily related to elaboration of RNA; work with labeled precursors is not ready for reporting at this time.

Proteinic brain hormone stimulated puffing at the index regions studied. There was some suggestion that the response was delayed, but insufficient testing was done to establish an obligatory delay in response at lower concentrations of hormone. Therefore, it is assumed that the response to this hormone is secondary and mediated through elaboration of ecdysones in the ring gland, although this is not firmly established by the data presented.

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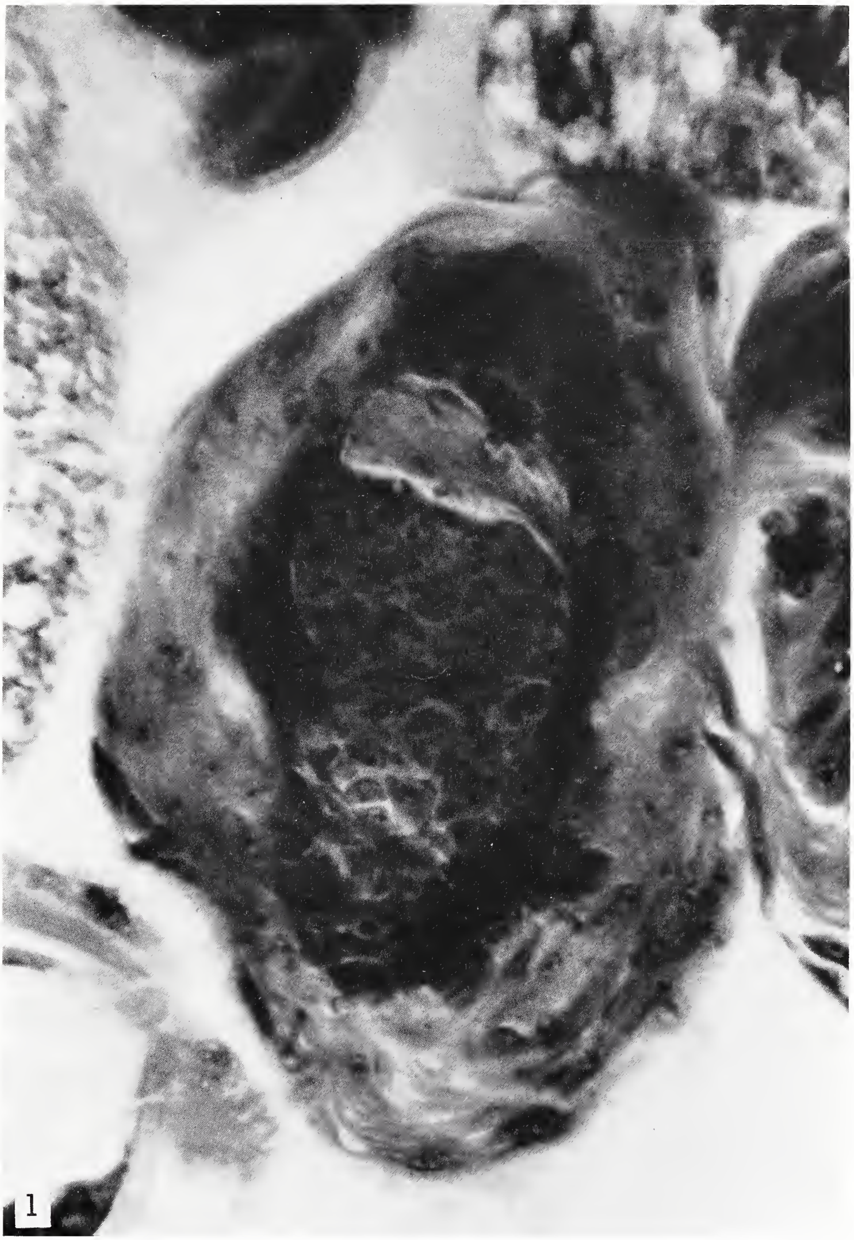

FIGURE 1.—Microscopic appearance of spontaneous melanotic tumors in *Drosophila melanogaster*. *Note* polygonal cells, fusiform stroma, and melanization beginning at periphery.

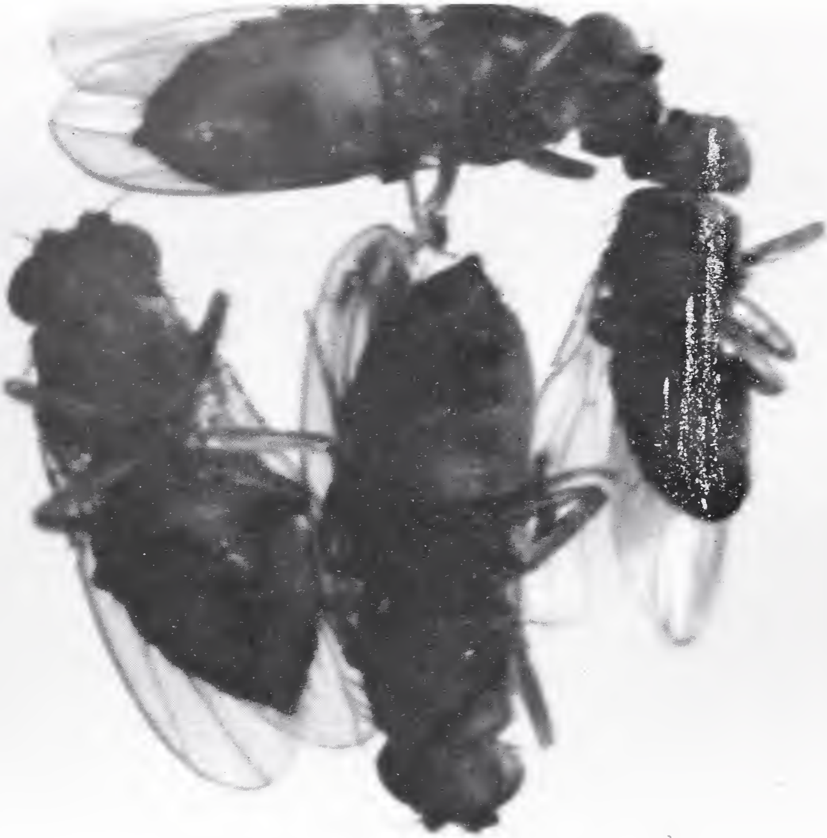

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FIGURE 2.—Gross appearance of melanotic detritus in imagoes.

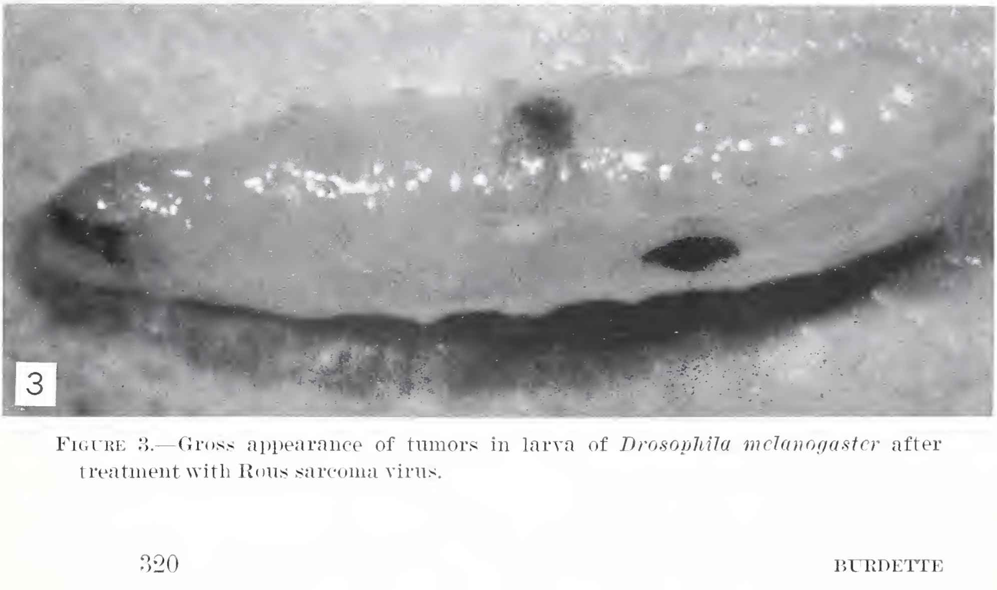

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FIGURE 3.—Gross appearance of tumors in larva of *Drosophila melanogaster* after treatment with Rous sarcoma virus.

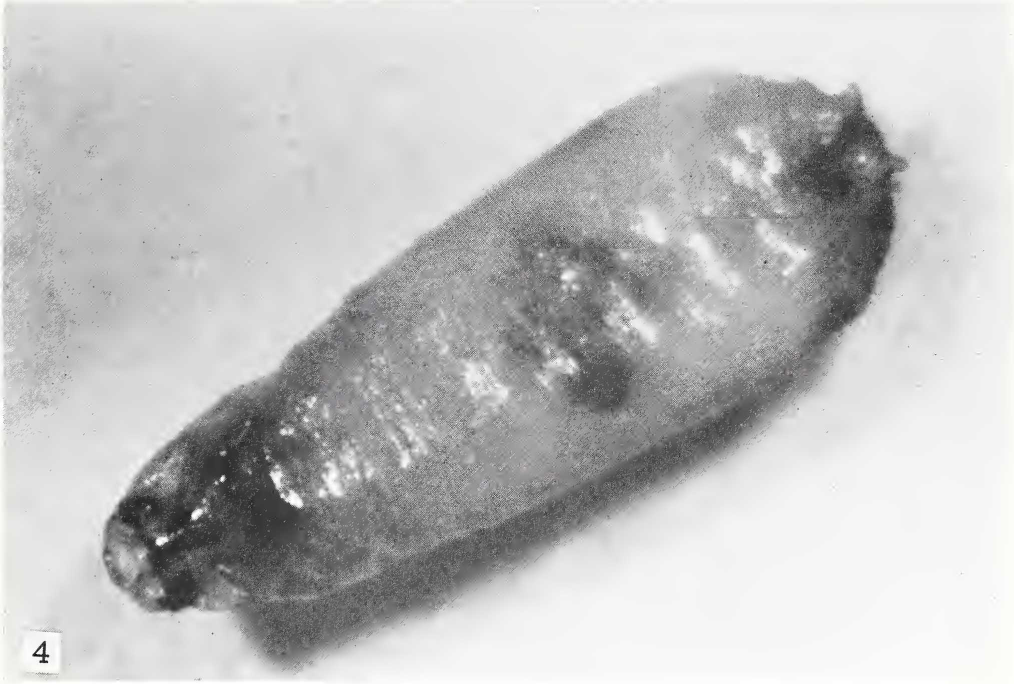
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Figure 4.—Gross appearance of tumor in pupa of *Drosophila melanogaster* after treatment with Rous sarcoma virus.

Hereditary Ovarian Tumors of *Drosophila melanogaster* [1]

ROBERT C. KING, *Department of Biological Sciences, Northwestern University, Evanston, Illinois 60201*

SUMMARY

Cytogenetic studies on *fused* and *female sterile* mutant genes that produce supernumerary ovarian cystocyte divisions in *Drosophila* are reviewed. The results are consistent with the hypothesis that each gene codes for a different polypeptide which forms a subunit of a dimeric mitogen. The normal mitogen functions to stimulate rapid divisions of cystocytes. The intermitotic period is so short that the cystocytes divide before doubling their birth volume and consequently diminish in volume as the divisions proceed. Mitosis is inhibited once the volume of a cystocyte falls below a critical minimum value (50 μ^3). Mutation at either locus produces an unstable mitogen, which results in a prolongation of the intermitotic period. Under such circumstances cystocytes grow sufficiently between divisions, so that the critical minimum volume required to shut off further mitoses is never reached.—Nat Cancer Inst Monogr 31: 323–345, 1969.

AN OVARY of *Drosophila melanogaster* consists of a parallel cluster of ovarioles, each of which is differentiated into an anterior germarium and a posterior vitellarium [*see* fig. 1 of (*1*)]. The vitellarium is composed of a series of interconnected egg chambers which lie in single file. Each chamber is more developed than the one anterior to it, and each contains an oocyte and 15 nurse cells surrounded by a monolayer of follicle cells [*see* fig. 2 of (*1*)]. The egg and its 15 nurse cells are fourth generation descendants of a single germarial cell called a cystoblast. The cells formed by the mitotic activity of a cystoblast are called cystocytes,[2] and it is within the anterior third of the germarium that such mitoses occur (*see* A, text-fig. 1).

The cystocytes generated from a single germarial cystoblast form a branching chain of cells connected by canals, each surrounded by a proteinaceous ring [*see* fig. 6 of (*1*)]. These cells can be characterized by the number of ring canals each contains. Two cystocytes (designated 1e

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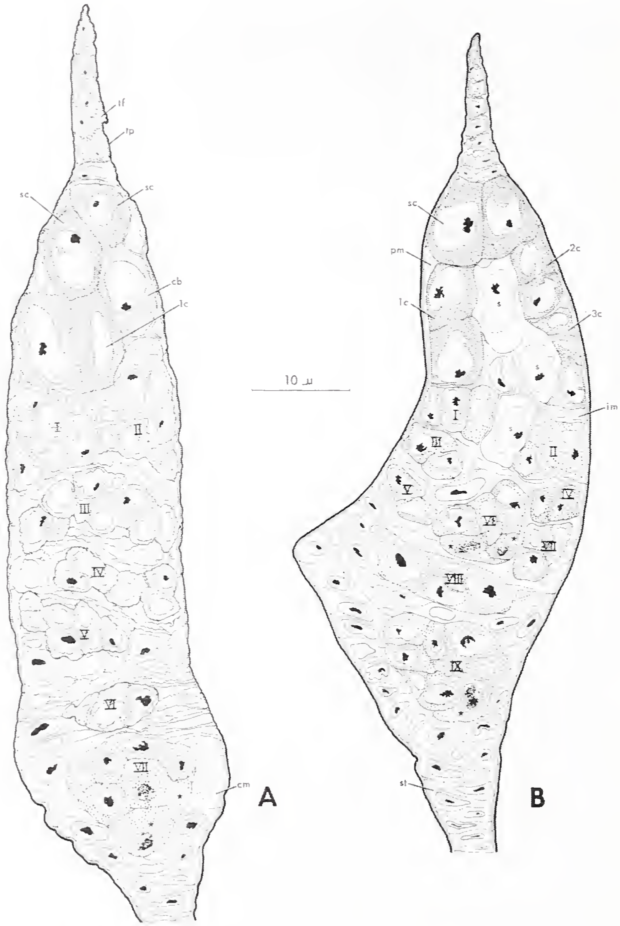
[1] Supported by grant GB 7457 from the National Science Foundation and grant E 408A from the American Cancer Society, Inc.

[2] The prefix *cysto* derives from the Greek *kystis* meaning bladder. We use it, however, in a different sense, that of cell aggregate. Ovarian cystocytes are in no way related to the class of hemocytes called cystocytes by some entomologists.

and 2e) are interconnected and each possesses 4 ring canals; two cells (3e and 4e) contain 3 canals, four cells (5–8e) contain 2 ring canals, and eight cells (9–16e) have but 1 canal each [*see* fig. 7 of (*1*)]. Cells 1e and 2e undergo a different type of nuclear differentiation from cells 3–16e. Since only these 2 cells enter meiotic prophase, they have been named pro-



TEXT-FIGURE 1.—Diagrammatic drawings of sections through *Drosophila* germaria subjected to electron miscroscopic analyses of serial sections: A) The fu^+/fu^+ germarium, B) the fu^1/fu^1 *germarium*. The terminal filaments point anteriorly in the insects. 1c, 2c, and 3c are first, second, and third generation cystocytes. cb, cystoblast; cm, cuboidal mesodermal follicle cell; im, invasive mesodermal follicle cell; pm, peripheral mesodermal cell; s, single subapical germ cell; sc, stem cell; st, stalk cell; tf, terminal filament; tp, acellular tunica propria. The normal germarium contains two single apical cells, three 2-cell clusters, one 4-cell cluster, one 8-cell cluster, and seven 16-cell clusters (I–VII). The *fu* germarium contains five single apical cells, five single subapical cells, three 2-cell clusters, one 4-cell cluster, one 8-cell cluster, and nine 16-cell clusters (I–IX). Cells of different types are given different amounts of stippling for contrast. In reality their electron densities are similar (*1*).

oocytes, while the remaining 14 are called pro-nurse cells. The production of fourth-generation cystocytes and their differentiation into pro-oocytes and pro-nurse cells begin during the pupal stage and continue throughout adult life (*2*). In each 16-cell cluster, however, 1 of the 2 pro-oocytes eventually switches to the nurse-cell developmental pathway (*3*).

OVARIAN TUMOR MUTANTS

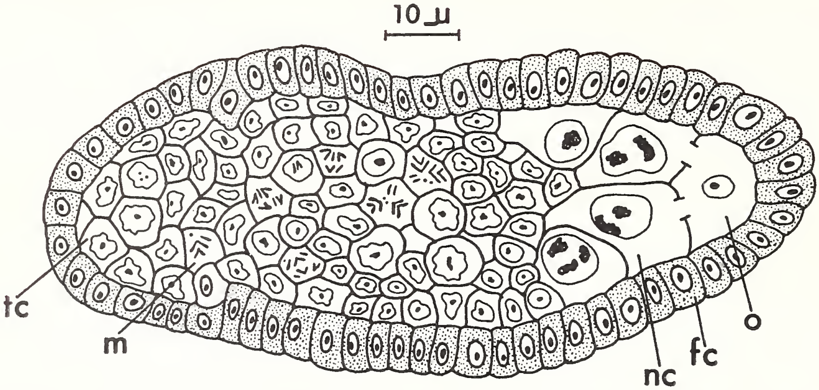
Two recessive mutations that render homozygous females sterile, because ovarian tumors are produced, have been extensively studied (*4*). These mutations are *fused* (*fu*) and *female sterile* (*fes*). The tumorous chambers seen in the ovarioles of homozygotes contain cells resembling germarial cystocytes. Such chambers grow slowly by mitosis and some eventually contain as many as 10,000 cells. While aneuploid, polyploid, and multipolar metaphases are sometimes observed, the karyotype of the tumor cells is generally normal. Cell division continues when tumors are transplanted to new hosts (*5*). However, the tumors do not invade nonovarian tissues and apparently have little effect on the viability of the donor or the host.

THE *FUSED* (*fu*) MUTATION

Drosophila melanogaster showing the *fu* mutation were first observed in 1912 by C. B. Bridges, who subsequently localized the recessive gene responsible at 59.5 on the linkage map of the X chromosome (*6*). The salivary chromosome locus was shown to be within the region bordered by 17B6 and 17F1 (*7*). The *fu* gene received its name from its effect on the morphology of the wing veins [*see* text-fig. 1 of (*8*)]. The fecundity of fu^1 homozygotes is only about 7% that of heterozygous *fu* females, due to a reduction in the number of ovarioles per ovary, the fusion of many adjacent, developing chambers, and, most important, the production of tumorous chambers. The frequency of such chambers rises with age (*9*). Where the fusion of a normal and a tumorous chamber takes place, the tumor cells may infiltrate the tissues of the normal-developing egg-nurse cell complex (text-fig. 2). In this sense *fu* tumor cells are invasive.

Effects on Embryogenesis

Young females homozygous for the fu^1 gene do produce 10 or 12 normal-appearing eggs, which develop only if they have been fertilized by sperm bearing the plus allele of *fu* (*10*). These eggs are apparently deficient in some vital compound, hereafter referred to as the *fu plus substance*, that can be manufactured subsequent to fertilization, if a plus allele has been contributed by the sperm. Counce (*11*), who studied the development of embryos produced by the fertilization of fu^1 eggs from homozygous fu^1 females by fu^1- or Y-bearing sperm, found that morphological abnormalities could first be seen in these embryos about 5 hours after fer-



TEXT-FIGURE 2.—A compound chamber from a *fu/fu* female. The chamber resulted from the fusion of adjacent chambers, the anterior one tumorous and the posterior one normal. fc, follicle cell; m, metaphase; nc, nurse cell; o, oocyte; tc, tumor cell.

tilization. These abnormalities included an asymmetrical development of the germ layers, which led to many ensuing developmental aberrations. Such embryos never hatched, but remained alive long after normal embryos had emerged as larvae. The *fu* eggs produced by heterozygous females develop normally, irrespective of the genotype of the sperm fertilizing them. King, Burnett, and Staley (*9*) suggested that the explanation for this maternal effect may be that quantities of the *fu plus substance* sufficient for normal embryogenesis have been incorporated into the ooplasm by the nurse cells, which have the maternal genotype and consequently possess the *fu* plus allele.

Multiple Alleles at the *fu* Locus

At least 10 independent instances of mutation at the *fu* locus (*12*) have been reported, and 7 of these mutant alleles were studied by Smith and King (*8*). All mutants were phenotypically identical with respect to wing morphology and the maternal effect on embryogenesis. However, the rates at which the homozygous females developed ovarian tumors differed significantly between certain alleles. The slowest rate was shown by fu^{59} and the highest by fu^{1} and fu^{62f3}. The other 4 alleles fell into 2 intermediate groups. To test for interactions between alleles, females heterozygous for all possible combinations of the 7 alleles were produced by appropriate crosses, and, in all 21 cases, when such heterozygous females were sacrificed at 8 days of adult age, their ovaries were found to contain tumors. An interesting observation emerged from a comparative study of the observed tumor frequencies. Six of the *fu* alleles are grouped into pairs because of similarities in the speed with which ovarian tumors develop. However, the 2 alleles in each group can be distinguished by their effect on tumor production when in combination with certain "test" *fu* alleles. Thus all mutant *fu* alleles so far studied are detectably different with respect to tumor formation, whereas none can be distinguished on the basis of their other phenotypic effects.

The rate at which ovarian tumors form in fu^1 and fu^{59} homozygotes differs by a factor of 6. By suitable crosses Smith and King (*8*) were able to show that this difference is allele-specific and not due to differences in the genetic backgrounds of the two strains. If reciprocal crosses are made between fu^1 and fu^{59} flies from isogenic lines, the fu^1/fu^{59} daughters produce tumors at a rate characteristic of the fu^{59} allele, whether it is contributed by the male or female parent. Thus, while the fu^{59} allele behaves dominantly to fu^1, there is no evidence for a maternal effect as there is in embryogenesis.

Gene Dosage Effects

It is possible to produce X chromosomes deficient for *fu* locus by recovering crossovers between specific inversions. One can then compare the tumor frequencies of *fu* homozygotes (fu^1/fu^1) and hemizygotes (fu^1/fu^-). The incidence in the former is half that in the latter (*13*). The incidence of tumors in fu^{59} hemizygotes is 10 times that found in homozygotes (*14*). Thus females carrying one dose of a given mutant allele form more tumors than do flies carrying two doses of the same allele. These findings suggest that both fu^1 and fu^{59} are producing a product, for, if both mutant genes were inactive, then two doses would have no more effect than one. It follows that the *fu* phenotype arises from the production of an abnormal gene product. Furthermore, in the case of the alleles with detectably different phenotypes (fu^1 and fu^{59}, for example), the mutant products must have different properties.

Temperature Effects on Tumor Incidence

Ovaries of 6-day-old fu^1/fu^1 females reared at 18°C throughout preadult and adult life have a tumor incidence one-tenth that of females of the same adult age reared at 25°C. In fact, the ovaries of 6-day fu^1/fu^1 females from the 18°C series have a tumor incidence equivalent to a 1-day-old fu^1/fu^1 female reared at 25° C. However, the ovaries of 6-day-old flies reared at 18°C are developmentally more advanced than those of day-old flies reared at 25°C (for example, they contain 5 times as many mature oocytes). Therefore the reduced tumor frequency in ovaries from *fu* flies reared in the cold is not due to a general retardation of ovarian development. Rather, the product of the *fu* locus apparently functions better at lower temperatures. Smith and King (*8*) suggest that the mutant product of the fu^1 gene is more stable at low than high temperatures. In more recent studies newly eclosed adults have been subjected to short exposures at more extreme temperatures (40°C for 20 min or 12°C for 2 hr). The flies were routinely maintained at 25°C. A week after treatment the incidences of ovarian tumors were compared between temperature-shocked and control ovaries. Heat shocks raised and cold shocks lowered the incidence of tumors.

Influence of the Y Chromosome Upon Tumor Incidence

Drosophila strains are available which contain fragments of Y chromosomes translocated to X chromosomes. By suitable crosses, females were produced containing a specific X-Y translocation and a normal X chromosome possessing a *fu* allele. A crossover between such chromosomes will produce an X chromosome containing *fu* and an attached Y fragment. Using such techniques, P. A. Smith (*14*) synthesized *fu* strains that may be symbolized as follows: $fu/fu.Y^S$, $fu/fu.Y^L$, and $fu.Y^S/fu.Y^L$. The symbols $fu.Y^S$ and $fu.Y^L$ represent X chromosomes carrying fu^1 and the short (Y^S) or long (Y^L) arm of the Y chromosome. A comparative study of the incidence of tumors in females of the karyotypes described above demonstrated that the addition of a Y chromosome substantially increased the tumor incidence. However, the increase in tumor incidence was due solely to the short arm of the Y. To see if this effect also applied to other *fu* alleles, a $fu^{59}.Y^S$ chromosome was synthesized, and the tumor incidences of $fu^{59}/fu^{59}.Y^S$ and fu^{59}/fu^{59} females of identical age were determined. The incidence of the former was 3.7 times that of the latter.

One noteworthy difference between Y^L and Y^S is that only Y^S contains a nucleolus organizer. Since the X chromosome also contains a nucleolus organizer, male and female *Drosophila* each possess two. However, the $fu^{59}.Y^S/fu^{59}$ and $fu.Y^S/fu$ females would possess three nucleolus organizers per nucleus. Ritossa and Spiegelman (*15*) have shown that the nucleolus organizer region of *Drosophila* chromosomes is the site where ribosomal RNA molecules are transcribed. A decision as to whether the possession of an additional nucleolus organizer is the cause of the accelerated production of ovarian tumors by *fu* females possessing the short arm of the Y chromosome requires further investigation.

THE FEMALE STERILE (*fes*) MUTATION

The recessive mutation, *fes*,[3] was discovered in 1929 by C. B. Bridges and localized at 5± on the linkage map of chromosome 2 (*6*). Homozygous males and females appear normal. Males are fertile; females sterile. When homozygous, the *fes* gene does not confer any special growth factor requirements on larvae, and homozygous *fes* females have normal viability (*16*). King, Burnett, and Staley (*9*) examined Feulgen-stained wholemounts of ovaries from such females of varying ages (5–30 days). The ovaries were found to be subdivided into a series of sausage-shaped masses of cells, and each mass was surrounded by a rather ill-defined follicular epithelium (text-fig. 3). The follicles were filled with thousands of undifferentiated tumor cells (fig. 2A). As the flies aged, the ovaries grew in volume because of an increase in the size and number of the tumorous chambers. Vitellogenesis never occurred within such chambers.

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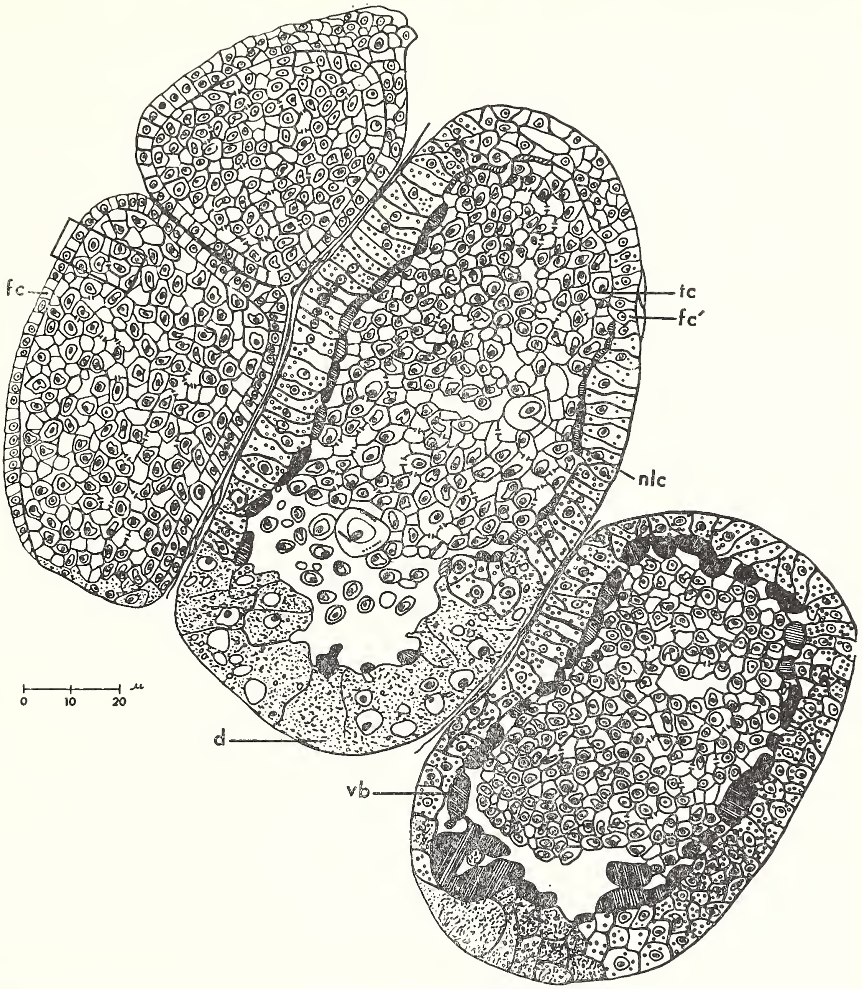
[3] In their recently published compendium of *Drosophila* mutants, Lindsley and Grell (*12*) have changed the designation of this mutant to *female sterile* (*2*) *of Bridges* (*fs* (*2*)*B*).



TEXT-FIGURE 3.—A drawing of four ovarian chambers from a 15-day-old female of genotype *S fes + + Alu lt/ + fes* dp^{tx} *Sp + +* reared at 25° C. *Note* numerous clusters of interconnected cells. Area enclosed in *rectangle* is shown at higher magnification in figure 1. d, degenerating cellular debris at the posterior pole of the chamber; fc, cuboidal follicle cell; fc^{1}, columnar follicle cell containing epithelial bodies; nlc, nurse-like cell; tc, tumor cell; vb, vitelline bodies. *See* (*18*) for further discussion.

The Ovarian Tumors of *fes*

Ovarian tumors from *fes* homozygotes vary in size between 1×10^3 and 1×10^6 μ^3 and contain between 10 and 10,000 cells. The smaller chambers drawn in text-figure 3 show the more typical situation where the follicle is filled with hundreds of cystocytes and surrounded by a layer of cuboidal follicle cells. The follicular epithelium is abnormal in that it is multilayered in some regions. The follicular epithelia of the larger chambers contain in several regions columnar follicle cells 5–10 μ thick. Vitelline bodies have formed in the intercellular space between these and the outermost layer of tumor cells. Thus the follicle cells synthesize vitelline membrane against tumor cells, if the oocyte is absent. Similar deposits of vitelline membrane have been seen in *fu* tumors (*14*).

In electron micrographs, *fes* follicle cells can usually be distinguished because their cytoplasm contains a higher concentration of ribosomes than tumor cell cytoplasm. Large carbohydrate deposits frequently accumulate in the follicle cell cytoplasm (fig. 1), but they have never been seen in the cells surrounding wild-type chambers. Moreover, this material does not accumulate in *fes* follicle cells when vitelline membrane has been secreted. Since the follicle cell is believed to play a major role in the secretion of the vitelline membrane (*17*), these deposits are believed to represent accumulations of a carbohydrate precursor material which is normally used in the synthesis of the vitelline membrane (*18*).

The nuclei of tumor cells are lobulated and their cytoplasm lacks endoplasmic reticulum. In these respects tumor cells resemble the cystocytes in the mitotically active region of the normal germarium. Within the interior of any large *fes* tumor are many degenerating cells containing ruptured nuclei, myelin figures, and mitochondria that have lost most of their cristae (fig. 1). This degeneration may result from the accumulation of toxic materials in the interior of large chambers.

If *fes* tumor cells are modified cystocytes, each should be connected to its sister cells by ring canals. These canals are visible in sections viewed under an oil-immersion objective (text-fig. 3). Electron micrographs of sectioned tumors show numerous ring canals, and spindle remnants are often seen protruding through the canals (fig. 1). In azure B-stained sections viewed under the light microscope, light-staining, dumbbell-shaped areas are often observed between adjacent tumor cells. Presumably these areas correspond to spindle remnants.

When light microscopic analyses were made of 12 serially sectioned chambers, about 70 instances were found of 3 or more tumor cells connected by ring canals (*18*). The maximum number of canals observed per tumor cell was 4. Thus the modified cystocytes of *fes* tumors seem unable to maintain more ring canals per cell than the maximum number normally found. Most tumors are made up of varying numbers of clusters of interconnected cystocytes. For example, one chamber when reconstructed from serial sections was found to contain 116 tumor cells. These were distributed among 14 clusters, each containing from 2–14 cells. Since the average number of cells per cluster is 8, it is reasonable to assume that *fes* tumor cells differ from true cystocytes in that they can detach from one another and so start independent, although adjacent, clones. The formation of a clone of cells from a cell which detaches from its companions is the first requirement of a metastasis. Furthermore, such cystocyte tumors will continue to grow when implanted in the body of a wild-type host (*5*).

Nurselike Cells in *fes* Tumors

Ovarian tumors from homozygous *fes* females sometimes contain cells that, on the basis of chromosomal morphology, resemble the endopolyploid nurse cells of normal egg chambers (fig. 2B). King, Sang, and Leth (*16*) found that the percentage of tumorous chambers containing such "nurse cells" rises as the residual genotype becomes more heterozygous. For example, the incidence for flies of genotype *S fes*+ +*Alu lt*/+*fes* dp^{tx} *Sp* + + is 10 times that of flies of genotype *fes Alu lt*/*fes Alu lt* (the genotype of the flies used in the earliest experiments). Females of the former genotype were used in all subsequent studies of nurselike cells.

The frequency of chambers with nurselike cells in the ovaries of flies reared at 25°C increases from 0.01–0.35 during the first week of adult life. During the next 2 weeks the frequency remains between 0.3 and 0.4 (*16*). The frequency of chambers containing nurselike cells plateaus at 0.9 for adults 15–21 days old reared during larval, pupal, and adult life at 18°C. About 40% of these chambers also contain tumor cells. However, in flies 1–3 days old, 95–97% of all chambers contain *only* nurselike cells (fig. 2B). Furthermore, in chambers containing nurselike cells, the number of such cells per chamber is 3 or 4 times greater in chambers from flies reared at the lower temperature (*19*). Thus lowering the temperature not only raises the incidence of nurselike cells, but changes the age-frequency distribution.

Fes chambers possessing nurselike cells fall into two classes: 1) those containing an assemblage of nurselike cells whose nuclei are similar in size, and 2) those whose nuclei vary greatly in size. There are 4 times as many class 1 nuclei as class 2 nuclei (*19*). Class 2 chambers often contain two or more clusters of nurselike cells with the nuclei of the cells in each cluster of roughly equivalent sizes. It is reasonable to assume that such chambers are compound; *i.e.*, they arise in situations where two or more clusters of cystocytes are incorporated into the same chamber during its formation in the *fes* germarium. Since chambers are also found which contain both nurselike cells and tumor cells, different cystocytes in the same chamber can behave in mutually exclusive ways.

In the 25°C series large tumorous chambers with a distinctive morphology appear in females about 6 days after eclosion. The interior of these chambers contains tumor cells, many of which are degenerating. Around the edge of the tumor cell mass are large numbers of nurselike cells (fig. 3). By the 9th day nearly every ovariole contains one chamber of this type. These chambers are located at the uterine end of the ovariole and are therefore the oldest chambers in the ovary. The terminal tumorous chambers in 2-day-old flies contain very few nurselike cells. Obviously most of the nurselike cells in the terminal chambers of 9-day flies have arisen from cystocytes which were actively dividing a week earlier. Therefore *fes* cystocytes can stop dividing and differentiate into nurselike cells while the chamber containing them moves through the adult germarium. It follows that not all (and perhaps none) of the *fes* tumor cells have lost the ability to differentiate into nurse cells (*18*).

Comparative Anatomy of *fes* and Wild-Type Nurse Cells

In wild-type ovaries, nurse cell nuclei and oocyte nuclei differ in that only the former contain chromosomes that undergo a series of endomitotic replications. The first presumably occurs in the germarium (about the time the oocyte doubles its diploid DNA content prior to entering meiotic prophase), and 7 or 8 more doublings occur within the vitellarium (*20, 21*). In the earliest egg chambers the homolgous chromosomes in each nurse nucleus are conjoined, and all chromosomes adhere at their centromeric regions (*22*). As the chromosomes replicate they continue to show somatic pairing and so become noticeably thicker. The nurse chromosomes then lengthen by uncoiling and start to fall apart into their constituent threads. However, the strands continue to adhere at their centromeric and telomeric regions. Since each nucleus contains the telocentric X, 2 large metacentric autosomes, and 1 small telocentric autosome, there are produced 5 large and 1 small DNA-containing masses per nucleus. Prior to the vitellogenic stages the replicating chromosomes dissociate completely, and the nucleus becomes filled with a jumbled mass of DNA-containing threads.

The nurse cells from both *fes* and wild-type ovaries can be easily identified at the light microscopic level on the basis of their nuclear morphology once their diameters reach 10 μ. In *fes* the "nurse" chromosomes also undergo endomitotic doublings, but the sister chromatids of the paired homologues often adhere with their chromomeres in register to produce banded polytene chromosomes (*16*). These are seen in nuclei between 15 and 30 μ in diameter. The lifespan of such chromosomes must be relatively short, since many are observed in various stages of degeneration. Spherical vesicles, which mark the places where cells containing banded chromosomes have degenerated, are commonly seen in flies more than 1 week old (*17*). King, Sang, and Leth (*16*) interpreted the falling apart of the wild-type nurse-cell chromosomes, which normally occurs prior to vitellogenesis, as being due to their intense synthetic activity. Thus if multiple cistrons in each chromosome become active, the uncoiling and functioning of these DNA segments in transcription might make pairing impossible. The fact that most chromosomes in *fes* nurse cells maintain their banded polytene configuration suggests that they are relatively inactive. According to Schultz (*23*) the X chromosome in *fes* nurse cells is quite diffuse in contrast to the autosomes. In *Drosophila* most of the genes determining female sexual phenotype are located on the X chromosome (*24*). One would therefore expect that in the ovary, a specifically female organ, the X chromosome, with a preponderance of genes coding for female-specific products, would be hyperactive. The uncoiled state of the DNA in the X chromosome of *fes* nurse cells supports this hypothesis.

INTERACTION OF *fes* AND *fu*

The *fes* gene behaves differently from *fu* in that *fes* does not produce abnormalities in any organ but the ovary. In terms of the ovarian effect, *fes* must act earlier in development, since tumors appear immediately upon the eclosion of *fes* females whereas in *fu* females tumors appear gradually as the adult ages.

By appropriate crosses Smith and King (*8*) were able to produce females containing both mutant genes in double dose (*fu/fu; fes/fes*). Such flies showed the *fu* wing phenotype. When ovaries were examined from females of age 1, 3, or 6 days, only tumorous chambers were seen. Thus *fes* is epistatic to *fu* in terms of ovarian phenotype.

The ovaries of female homozygous for *fu* and heterozygous for *fes* (*fu/fu; fes/+*) had a higher incidence of tumors than *fu/fu; +/+* females of the same age. Thus the "recessive" *fes* gene can be detected in the heterozygous condition by its interaction with *fu*.

TRANSPLANTATION EXPERIMENTS

King and Bodenstein (*5*) have shown in the case of *fu* and *fes* that ovaries genetically destined to become tumorous do so even when they are transplanted to a normal abdomen. Normal ovaries transplanted to the abdomen of females homozygous for such tumor genes do not become tumorous. Therefore there is no evidence for diffusible tumorigenic agents as initiating factors in the development of the ovarian tumors characteristic of *fu* or *fes*. If *fu* or fu^{59} ovaries are implanted into an abdomen of a fly homozygous for the normal *fu* allele, the implanted ovaries develop tumors at the rate characteristic of each allele (*25*). Thus the rate at which tumors appear in such ovaries is a genetic characteristic and is not affected by the extraovarian environment.

King, Aggarwal, and Bodenstein (*26*) have made a comparative cytological study of the corpus allatum-corpus cardiacum complex of wild-type and *fes* adult females. They conclude that a secretion is continually synthesized and stored by the corpus allatum cells of female *Drosophila melanogaster*, which as a result of their *fes* genotype lack oocytes. The storage of the product is accompanied by degenerative changes in the cells of the corpus allatum. The product is postulated to be a hormone influencing vitellogenesis. The cortical cells of the corpus cardiacum also produce a secretion, which is stored in the case of *fes*. Again storage is accompanied by degenerative changes [contrast figs. 3 and 11 (*26*)]. If an immature wild-type ovary is implanted in a *fes* female, vitellogenesis takes place and is accompanied by the restoration of the corpus allatum-corpus cardiacum complex to its normal morphology. Thus it is not correct to say that *fes* affects only the ovary, but its effects on the corpus allatum-corpus cardiacum complex are secondary and can be cured.

DISCUSSION

The finding of clusters of identical mutations among the progeny of individual female *Drosophila treated as adults with ionizing radiation* constitutes conclusive evidence for the presence in the ovary of stemline oogonia, *i.e.*, mitotically active germinal cells that produce a continuing supply of cystoblasts (*3*). Koch and King (*20*) observed one or two large cells in each adult germarium adjacent to the terminal filament (text-fig. 1B) and suggested that these are stem cells which generally divide in such a plane that one of the two daughters is discharged posteriorly into the germarial lumen while the other continues to adhere to the blind, apical end of the germarium. The freed cell presumably behaves as a cystoblast, whereas the attached cell continues to function as a stemline oogonium.

If the volumes of germ cells from wild-type germaria are determined, it is found that each newly formed fourth generation cystocyte has a volume between 30 and 50 μ^3, which is only about one-fifth the volume of an apical stem cell. Furthermore, fourth generation cystocytes do not initiate further growth until they reach the most posterior region of the germarium about 3 days later. These observations led Koch and King (*20*) to suggest that cell volume functioned as the cue that led to the termination of cystocyte mitoses. Cystocytes whose volumes dropped below 50 μ^3 were postulated to receive the stimulus to differentiate into prooocytes or pro-nurse cells, and having done so, their ability to undergo further division once their growth started again on reaching the vitellarium was lost. This line of reasoning suggests that within the germarial lumen a cystoblast or a cystocyte undergoes mitosis once it reaches about 1.3 times its birth size. Under such a model, a mitotically suppressed fourth generation cystocyte eventually would be produced with a volume about 18% that of its ancestral cystoblast. On the other hand, the apical position of the stem cell would somehow allow it to double its volume between mitoses. Under such circumstances these cells would never cease dividing, because the critical minimum volume would never be reached regardless of the number of divisions.

Stem cells and cystoblasts also differ in that the former undergo complete cleavage, whereas the cytokinesis of cystoblasts is incomplete, resulting in chains of cystocytes connected by ring canals. During the larval stage only oogonia showing the stem cell type of cleavage are found. These oogonia divide during the larval stage and by the time of puparium formation sufficient cells have been formed to provide each differentiating germarium with 4–7 oogonia. The cystocyte divisions begin late in the prepupal period, and thus the signal resulting in the transformation of oogonia to cystoblasts coincides with, and may depend on, the same hormonal stimulus that causes metamorphosis (*2*). It is simplest to assume that ovarian metamorphosis results in the transformation of *all* oogonia. These henceforth behave as cystoblasts during mitosis, provided they do not reside in the region of the terminal filament. The mechanism which prevents apical oogonia from undergoing the cystocyte type of divisions is not known, but presumably involves some interaction between the apical oogonia and the neighboring cells of the terminal filament (text-fig. 1A). These cells show signs of secretory activity at the time of the oogonial transformation [fig. 6 of (*2*)].

Under the minimum volume hypothesis, any factor that leads to an accelerated synthesis of cytoplasm between divisions or to a long interval between divisions (while the synthesis of cytoplasm continues at a normal rate) would tend to keep cystocyte volumes from falling below the 50 μ^3 limit. Such factors would stimulate supernumerary divisions. Since supernumerary cystocyte divisions are one of the most characteristic effects of the *fes* and *fu* genes, their wild-type alleles may code for proteins that function in mitotic control. Perhaps the mitosis of oogonia requires a stimulatory compound or *mitogen* whose synthesis is controlled by fes^+ and fu^+.

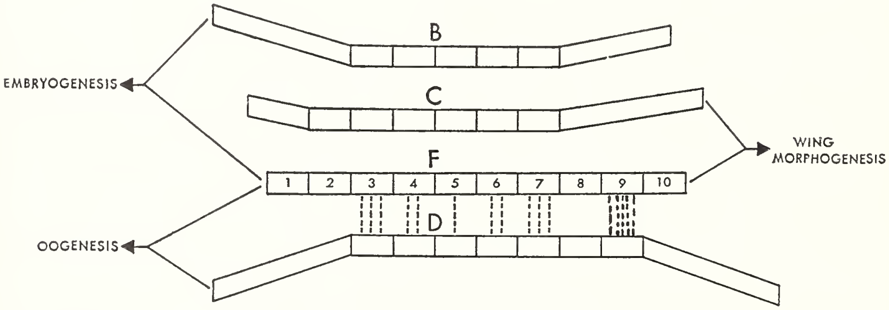
Fu, however, is an example of a gene with pleiotropic effects. In all *fu* alleles studied, the production of ovarian tumors was accompanied by an alteration in wing-vein morphology. Also, the maternal effect in which *fu/fu* embryos develop normally when the mother is *fu*/+ (but abnormally if she is *fu/fu*) has been found in all the alleles examined. Thus this syndrome (abnormal wing venation, ovarian tumors, and a maternal effect on embryogenesis) characterizes each of the *fu* alleles studied. Current thinking concerning transcriptions of the messages encoded in DNA is in terms of one cistron specifying one specific polypeptide (*24*). The question arises as to how one such polypeptide could affect developmental processes as diverse as embryogenesis, wing morphogenesis, and oogenesis.

Modern knowledge concerning the interaction of different proteins to form heteropolymers makes possible the construction of a hypothesis that can explain *fu* pleiotropy. The human hemoglobins (*27*), and enzymes exemplified by the vertebrate lactic dehydrogenases (*28*), are examples of biologically important heteropolymers. In hemoglobin, the α, β, and γ polypeptides are coded by nonallelic genes. *Alpha* chains form heteropolymers with γ chains during fetal development. Shortly after birth, γ-chain synthesis is suppressed, β-chain synthesis is activated, and α-β heteropolymers become the predominant form of hemoglobin. Thus in a given tissue a specific polypeptide can combine with another polypeptide at one developmental stage and with a different one at a subsequent stage, forming functional heteropolymeric proteins in each case.

It has been demonstrated for the lactic dehydrogenases that the active enzyme consists of tetramers containing either identical or different subunits. At least two gene loci are involved: one coding polypeptide A, the other coding polypeptide B. The tetramers A^4B^0 and A^0B^4 can be dissociated *in vitro*, and, on being mixed, the monomers recombine to form the tetramers A^3B^1, A^2B^2, and A^1B^3 (*29*). All five tetramers are also found *in vivo*. They catalyze the same reaction, but differ in ways which presumably have functional significance. The data concerning the lactic dehydrogenases demonstrate that a given polypeptide can combine with identical polypeptides in some tissues and with different polypeptides in other tissues, all at the same stage in development.

After a single mutation in *Escherichia coli*, the enzymes aspartokinase and homoserine dehydrogenase show a modified allosteric inhibition by threonine. The molecular masses of both enzymes, as determined by gel filtration, are altered to the same degree. Reversion results in a simultaneous recovery of both enzymatic activities. The above facts are interpreted as evidence that the two functionally different proteins are heteropolymers sharing a common polypeptide chain coded by a single cistron (*30*). Thus it appears that the same polypeptide chain may sometimes be found in different enzymes functioning concurrently in the same cell. If one cistron can specify a polypeptide which forms a subunit of two or more functionally different heteropolymers, mutation of the cistron will generate pleiotropy.

Let us consider the hypothetical product formed by the *fu* plus locus and suggest that it forms heteropolymers with other hypothetical polypeptides during the various developmental processes (text-fig. 4). The numbers 1–10 represent amino acids coded by the respective nucleotide triplets. It is postulated that the *fu* plus cistron produces a single polypeptide (represented by F). It is further hypothesized that in producing each characteristically different phenotype the polypeptide product interacts and forms heteropolymers with a different specific polypeptide. Thus the F substance forms dimers with a specific polypeptide coded by a different cistron (the B locus), and the BF dimers function during embryogenesis. During metamorphosis the B locus is turned off and another locus (C) is turned on. It produces a specific polypeptide (represented by C) which interacts with F to form CF dimers that function in wing development. Finally, another gene (the D locus) is turned on as the B and C loci are turned off, and FD dimers are produced which function as mitogens during ovarian development. In the model, amino acids 3–7 in the F polypeptide would make up a large pairing site, which functions to bind F to any



Text-figure 4.—A model devised to explain the pleiotropic effects of the *fused* gene. F indicates the polypeptide product of the fu^+ gene and B, C, and D hypothetical polypeptides with which F forms heteropolymers during embryogenesis, wing development, and oogenesis, respectively. Linearly arrayed *rectangular blocks numbered 1–10* represent the arbitrarily selected number of amino acids of which F is composed. The amino acid sites at which B, C, and D pair with F are also indicated by similar rectangular blocks in register with specific F segments. The strength with which amino acids residing in the F–D pairing segments interact is represented by the number of *dashed lines* connecting any two. The phenotypic result of amino acid substitutions at each of the positions 1–10 in the D or F polypeptide is indicated in the chart *below the diagram* (*8*).

of the three different polypeptide chains. Amino acid #2 would be involved only in the pairing site of F with C, and amino acid #9 would constitute a site essential to the pairing of F with D.

Amino acid substitution at position	Phenotypic effects				Mutant alleles represented
	Embryogenesis	Wing	Ovary	Tumor incidence	
F1	Normal	Normal	Normal	Nil	
F2	Normal	*fu*	Normal	Nil	
F3	Abnormal	*fu*	Tumorous	+++	fu^{1}
F4	Abnormal	*fu*	Tumorous	++	fu^{ff}
F5	Abnormal	*fu*	Tumorous	+	fu^{59}
F6	Abnormal	*fu*	Tumorous	++	fu^{57a}
F7	Abnormal	*fu*	Tumorous	+++	fu^{62f-3}
F8	Normal	Normal	Normal	Nil	
D9	Normal	Normal	Tumorous	++++	*fes*
F10	Normal	Normal	Normal	Nil	

We can now consider the result of an amino acid substitution in each of the 10 positions. As indicated in text-figure 4, a substitution in amino acid #2 would cause the FC dimer to be unstable and result in a *fu* wing phenotype (but ovarian development and embryogenesis would be normal). Such a case has not yet been observed. However, such an occurrence would be rare if this pairing site made up but a small portion of the molecule. Since the only individuals examined were those with *fu* wings, a substitution in amino acids 8 or 9 would not have been detected. An amino acid substitution in any one of the positions 3–7 would result in the subsequent production of unstable FC, FD, and FB dimers, which result, in turn, in wings of *fu* phenotype, ovarian tumors, and embryonic lethality (if the mother is *fu/fu*). The *fu* alleles that have been studied all show this syndrome. If it is assumed that relatively strong intermolecular bonds are normally formed at sites 3 and 7 when F pairs with D, substitutions of the amino acids at either of these sites would be expected to result in a rather extreme ovarian abnormality (as in *fu* and fu^{62f3} homozygotes, which are characterized by tumor production at a rapid rate). In the model, the bond formed by the amino acid at position 5 is relatively weak. Thus a substitution at position 5 would be expected to result in a less extreme ovarian abnormality (as in fu^{59} homozygotes, which show a very low rate of tumor formation). The mutant FD dimer would still be relatively stable since the strong binding sites are not modified.

The model presented here readily explains examples of pleiotropy, which are difficult to understand if one supposes that the gene in question has a single product and that the product, in turn, has a single function resulting in a single developmental event which, if mutant, finally produces manifold, but related, abnormalities. It must be admitted that the model is highly speculative, and that with a little ingenuity equally successful models could be devised with completely different properties. However, it does seem logical to suggest that during evolution a given polypeptide may function first as a monomer "A," later as a homodimer "AA," still later as a heterodimer "AB," and eventually acquire the ability to also form dimer "AC." In fact something of this sort has happened during the evolution of the hemoproteins (*27*).

Interaction of the *fu* Alleles in Ovarian Development

When a *fu* allele characterized by a low tumor incidence (like fu^{59}) is crossed with one characterized by a medium or high incidence (like fu^{57a} and fu^{1}, respectively), the compound individual generally has a low tumor incidence. In such cases two classes of FD dimers would be produced, one with an F3 substitution and the other with an F5 substitution; for example, *see* text-figure 4. The latter dimer would be by far the most stable and it therefore would mask the production of the phenotype characterizing the former dimer.

A cross between two alleles having high rates of tumor production resulted in a compound female (fu/fu^{62f3}) characterized by a low incidence of tumors. This finding may be explained by the assumption that the polypeptides produced by the two alleles can fragment and recombine in the cytoplasm, and that certain recombinant polypeptides have the normal amino acid sequence. Much the same argument has been used to explain complementation in *Neurospora crassa.* Two mutants lacking detectable adenylosuccinase activity exhibited when combined in heterocaryons, adenylosuccinase activity up to 25% that of the wild type (*31*). When the inactive extracts of the two mutants were mixed, enzyme activity could also be detected (*32*).

In one cross of a strain of flies characterized by low tumor incidence with one intermediate in tumor production, the compound females (fu^{59}/fu^{ff}) had a high rate of tumor production. Postulating the formation of dimers between the polypeptides produced by these two alleles may explain this finding. Thus, the affinity of the products F′ and F″ of the two different mutant alleles could be greater for each other than for D. Therefore, more F′F″ dimers would be produced than F′D or F″D dimers. Either F′ or F″ alone would form F′D or F″D dimers, respectively, and result in more normal ovarian behavior than is found when F′ and F″ are both produced.

Origin of the D Polypeptide

The question now arises as to the origin of the postulated D polypeptide. It has been demonstrated that the *fes* gene is epistatic to *fu* and that a *fu/fu; fes/+* female has a higher rate of tumor production than a *fu/fu; +/+* female. This increase in tumor production resulting from the presence of *fes* in the heterozygous state indicates the possibility of the fes^{+} product being the D polypeptide of the model. Both genes have been shown to be temperature-sensitive, females homozygous for either gene having a more normal ovarian morphology at 18 than at 25°C. This finding could indicate that the dimer having either D or F in the mutant form is more stable at 18°C and therefore functions more nearly like the DF dimer produced in the ovaries of wild-type females. The *fes* gene is presumed to function only in the ovary, since no phenotypic effects have been observed in *fes/fes* flies aside from the production of ovarian tumors. A number of *fu* alleles have been studied, but none results in as extreme an ovarian abnormality as in *fes/fes* females. Only one *fes* allele has been found and studied. Possibly in this particular case a small but very important site on the *fes*$^+$ cistron has been altered. The DNA codon for amino acid D9 (text-fig. 4) would be an example of such a site. It will be interesting to see if one could construct, through intragenic recombination, a *fu* allele with two mutated sites resulting in the production of ovaries that are completely tumorous at eclosion.

Mechanisms of Action of FD Dimer

If the FD dimer functions in oogonia as a mitogen, the mutant mitogen may be less stable and therefore generally present in lower concentration. As a result, the period between mitoses would be prolonged in mutant cystocytes. A prolonged intermitotic period would be reflected morphologically in the germarium by the presence of clusters containing fewer than 16 cystocytes in the region of the germarium in which only 16-cell clusters are normally found. This is exactly the situation observed in the *fes* germarium (*18*). Furthermore, the rate at which chambers enter each vitellarium is only about a third that observed for wild type. The *fu* germarium contains more single cells and more 16-cell clusters, and a smaller portion of the 16-cell clusters have arranged themselves into a single-file order in the mutant than in the wild-type germarium. Large, single, subapical cells are present solely in the *fu* germarium (*cf.* text-figs. 1A and 1B). Such single cells are thought to be "slow dividers," since they reside in a region occupied by cells that have divided 1, 2, 3, or 4 times (first, second, third, and fourth generation cystocytes). These cells presumably continue to grow and divide at such relative rates that the progeny cells remain at volumes above the critical minimum value. Such mutant cystocytes never receive the cue to stop dividing, and so give rise to the ovarian tumors characteristic of *fu*. Thus in *fu* and *fes* we have a paradoxical situation where supernumerary divisions accompany abnormally long intermitotic times. The hypothesis suggests the prolonged mitotic time is caused by a defective FD dimer and results in cystoblasts that continue dividing because they never reach the minimum volume required to shut off further mitoses.

The finding that newly emerged *fu* flies are free of ovarian tumors, but that they appear in ever-increasing frequency as the female ages, can be explained by assuming that the cystocytes enter the adult period with an excess of the mutant F′D dimer due to storage of that mitogen produced during the pupal period. Once this surplus is eliminated, further synthesis does not meet the demand. Storage of the FB dimer (text-fig. 4) accounts for the maternal effect upon embryogenesis characterizing *fu*.

Lowering the temperature of the insect causes many mutant cystocytes to cease dividing and to differentiate. This effect is most easily explained by assuming that the mutant mitogen is more stable at low temperature. In *fes* females reared throughout the larval, pupal, and adult stages at 18°C, the incidence of chambers containing only nurselike cells falls from a value of about 0.97 during the first day of adult life to 0.28 a week later. During the same interval, ovaries from flies reared at 25°C show frequencies that vary randomly between 0.01 and 0.10 (*18*). These results may be explained by assuming the mutant FD′ mitogen is much more stable at 18 than at 25°C and therefore appreciable storage of mitogen produced during the pupal period can occur only at 18°C. The stored mitogen is used up during the first week.

The fact that *fu* and *fes* ovaries behave autonomously in transplantation experiments (*5*, *25*) leads to the conclusion that the FD dimer is nondiffusable.

In *fes* ovaries, cystocytes at the periphery of tumors with necrotic centers differentiate into "nurse" cells (fig. 3). Here toxic materials from degenerating cells may cause a slowed growth during the intermitotic period which allows cells upon division to reach the required minimum volume. In the case of *fu*, an added Y^s is known to stimulate tumor production. Here an added nucleolus organizer may result in an increase in the cytoplasmic concentration of ribosomes. This in turn may increase the rate of synthesis of cytoplasm during the intermitotic period and so prevent cystocytes from reaching the minimum volume required to stop dividing.

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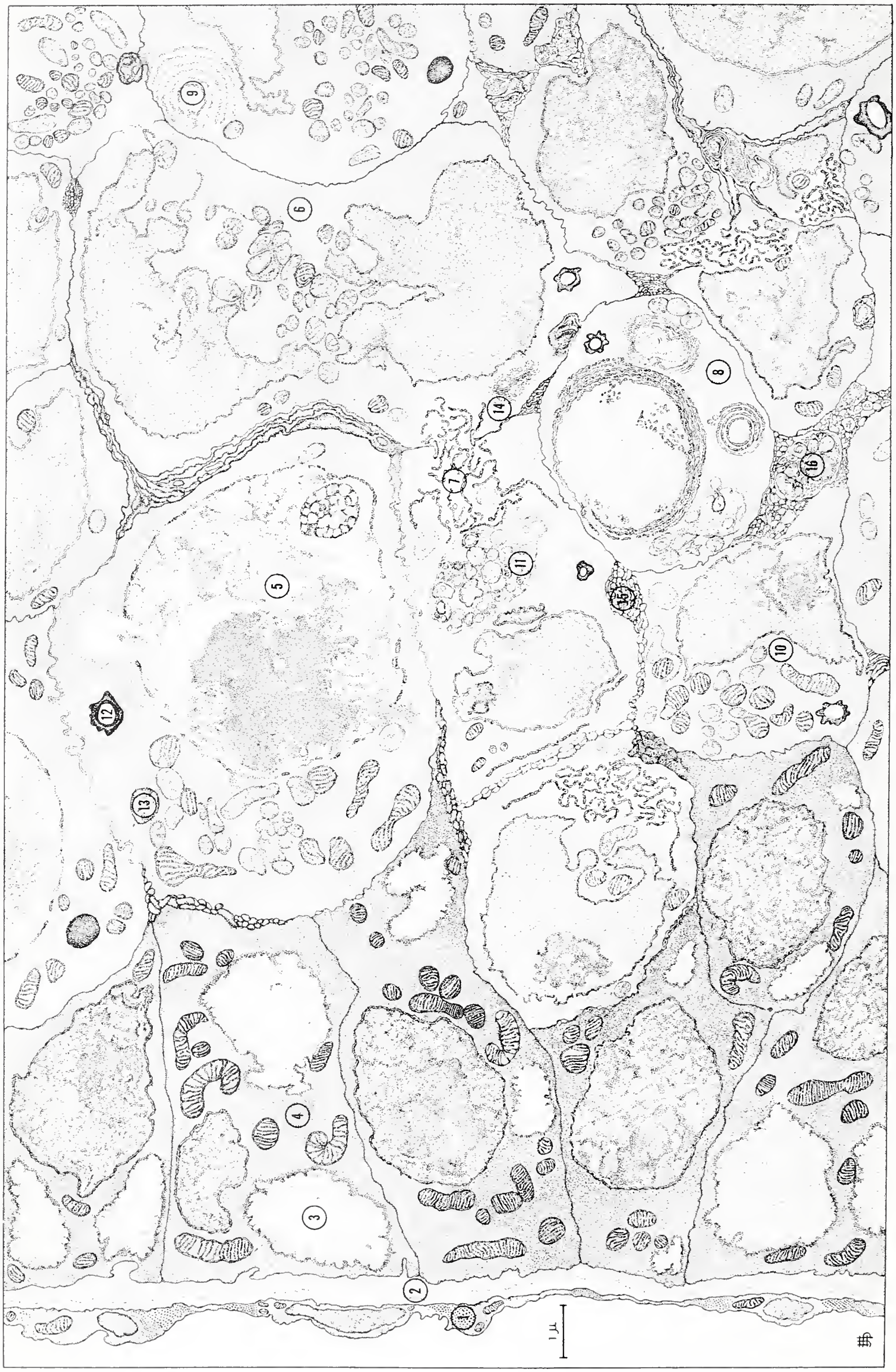
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FIGURE 1.—Composite, made from about 50 electron micrographs, shows a portion of a *fes* tumorous chamber (*see* text-fig. 3 for orientation). 1) epithelial sheath. *Note* parallel clusters of myofilaments in the cytoplasm, the nuclei, and the mitochondria, 2) the tunica propria, 3) a carbohydrate deposit, 4) a follicle cell containing mitochondria and a nucleus, 5) the nucleus of a nurselike cell containing a multicompartmented body, 6) a tumor cell at telophase. *Note* that this cell is connected by a ring canal to an adjacent tumor cell, 7) spindle remnants, 8) degenerate cell, 9) annulate lamellae, 10) tumor cell, 11) a cluster of phagocytized cellular debris, 12) a lipid droplet with its center extracted, 13) degenerating mitochondria, 14) Golgi apparatus, 15) microvilli between two tumor cells, 16) cellular debris in the intercellular space between tumor cells. *See* (*17*) for further details.

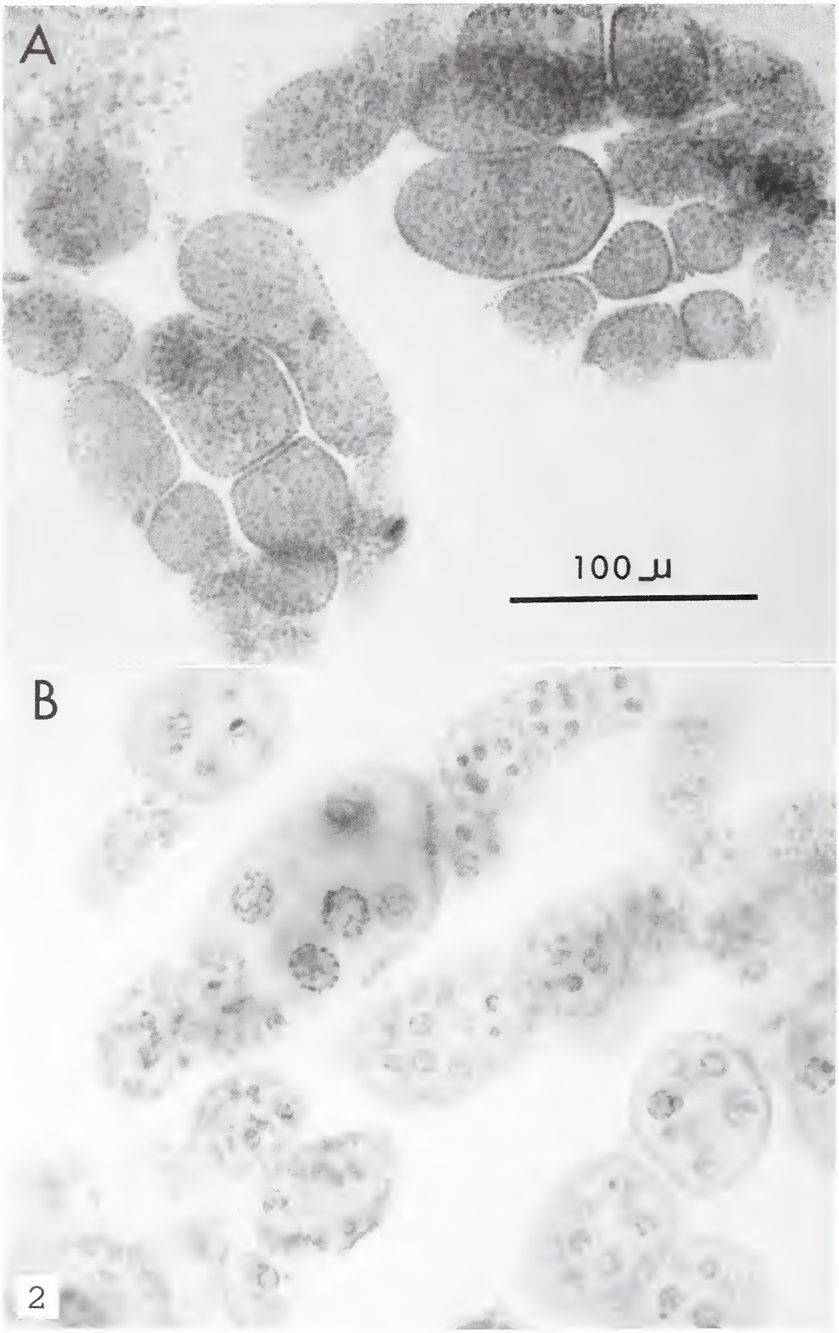

FIGURE 2.—Feulgen-stained, ovarian wholemounts from 2-day-old females of genotype *S fes* ++ *Alu lt*/ + *fes* dp^{tx} *Sp* ++ reared at one of two temperatures. A) Only tumors are seen in the ovary from the 25°C series. B) Only chambers containing nurselike cells are seen in the ovary from the 18°C series.

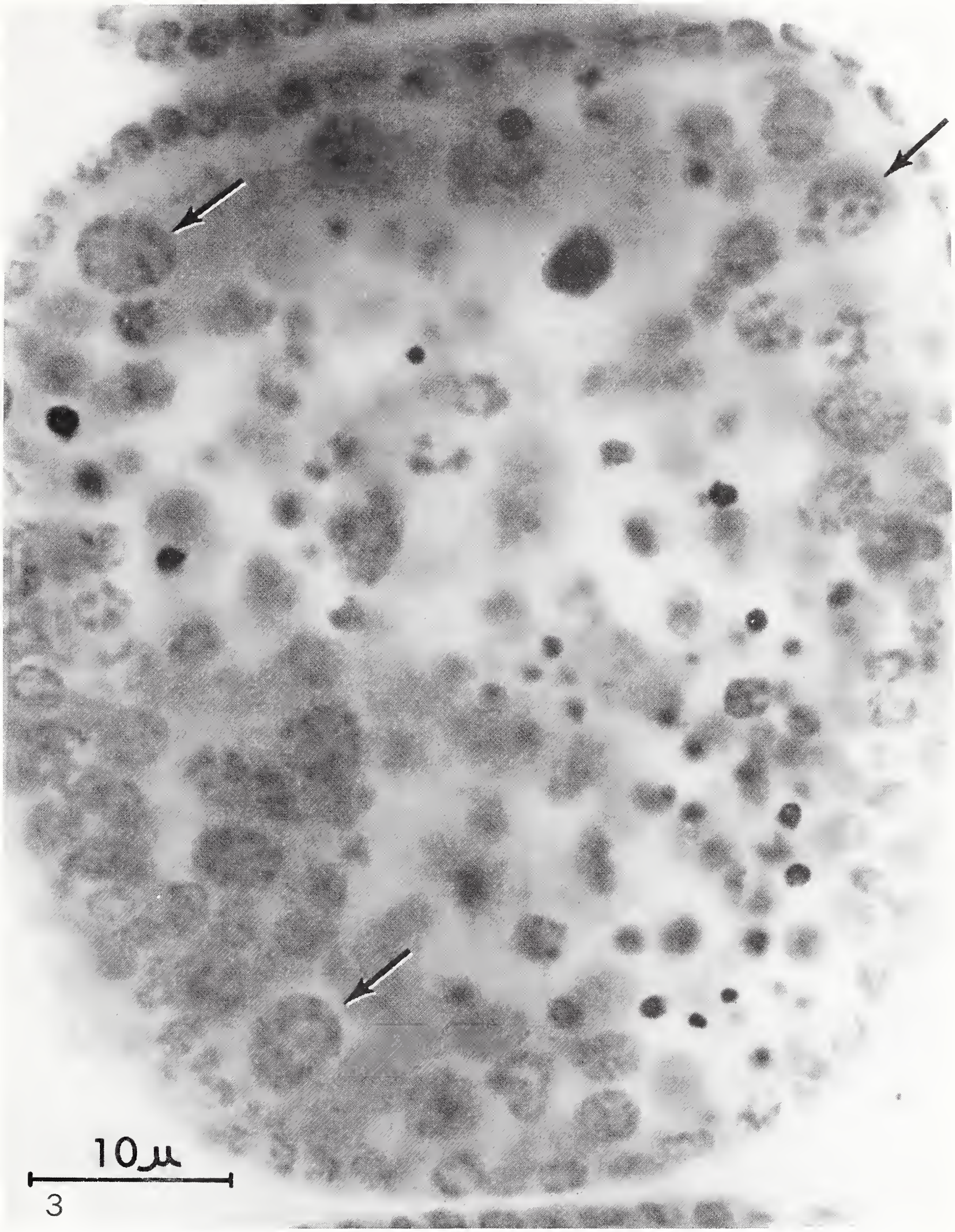

FIGURE 3.—Photomicrograph of a large, Feulgen-stained, tumorous chamber from an 8-day-old *fes* female from the 25°C series. Chamber contains numerous stained blobs that represent degenerating tumor cells. *Arrows* point to representative nuclei belonging to cells located around the edge of the chamber that appear to be differentiating as nurselike cells. *See* (*18*) for further details.

Control of Oocyte Formation by *Female-Sterile (fes) Drosophila melanogaster*[1]

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It was pointed out in my contribution this morning that under appropriate conditions *female-sterile* (*fes*) females will produce chambers in which most cystocytes, instead of continuing to divide, differentiate into "nurse" cells. While the number of "nurse" cells per chamber varies between 1 and 40, the average chamber contains about 10. Their nuclei often reach the maximum volume seen for wild-type nurse cells, but unlike these cells, *fes* "nurse" nuclei contain banded polytene chromosomes and a large spherical nucleolus (*cf.* figs. 1C and 1D in text-fig. 1). About 2% of such *fes* "nurse" chambers also contain an oocyte that can undergo vitellogenesis (*cf.* figs. 1A and 1B in text-fig. 1). After an analysis of egg chambers containing subnormal and supernormal numbers of nurse cells, we concluded that vitellogenesis proceeds with as few as 10 and as many as 30 nurse cells.

The three-dimensional morphology of 13 *fes* chambers containing nurse-like cells was determined by an analysis of serial sections. The data are presented in the accompanying table. Note that all oocyte-deficient chambers lack cystocytes possessing 4 ring canals, and that all cystocytes possessing 4 canals differentiated into oocytes. These findings support the hypothesis developed earlier by Elizabeth Koch, Patricia Smith, and me (*1*, *2*) that wild-type cystocytes possessing less than 4 ring canal rims are programmed to develop as nurse cells, while those with 4 become oocytes.

Cystocytes characteristically divide to form a branching chain of cells. In a normal chamber 25% of the cells (the cells with 3 or 4 canals) are sites where such branching occurs. The *fes* cystocytes in the 13 chambers just catalogued behave similarly, since 21% of these cells contain 3 or more canals. Note also that approximately 40% of the cystocytes in the *fes* chambers were binucleate, suggesting a retarded synthesis of plasma membrane. Microscope slides illustrating these points have been donated to the specimen collection of the Registry of Tumors in Lower Animals.

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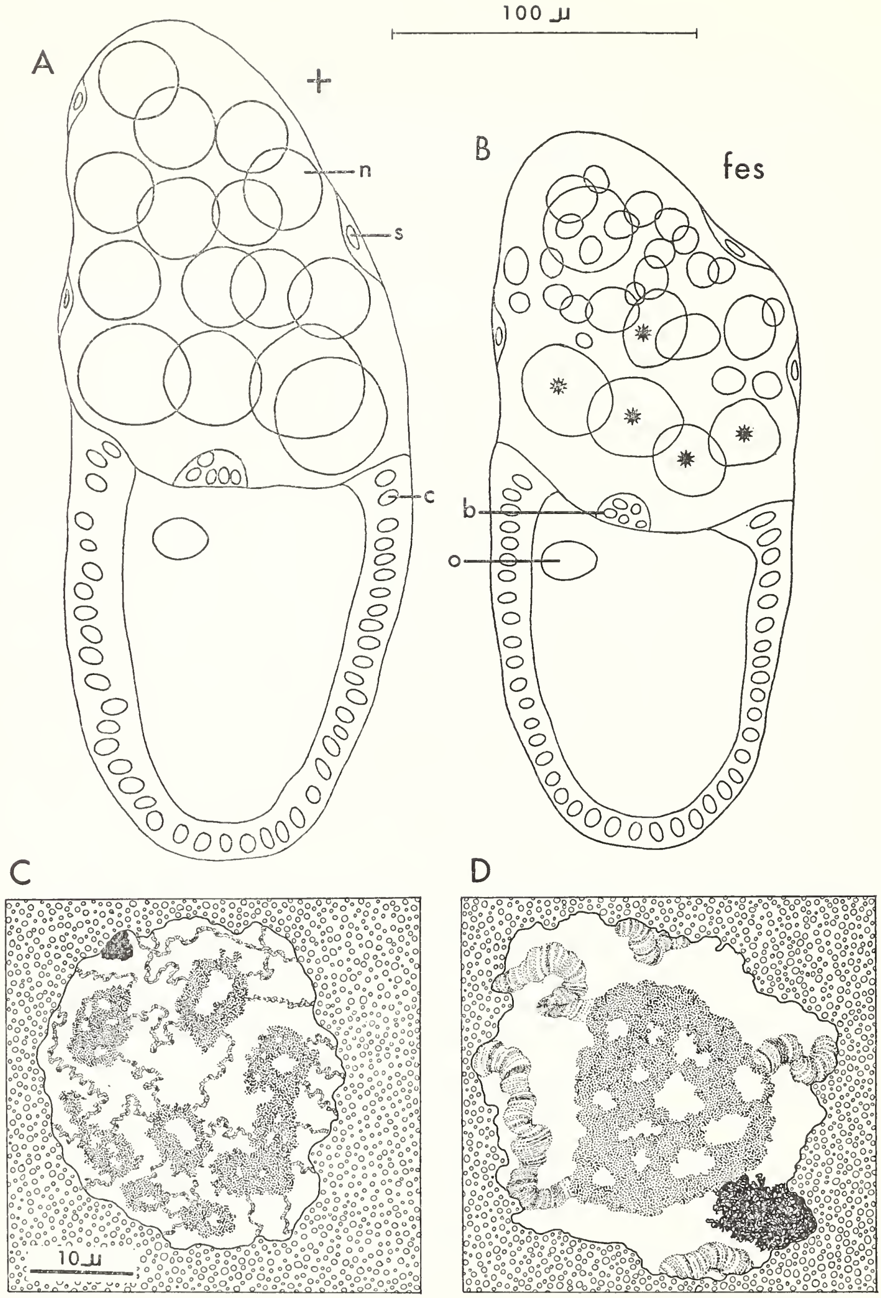
[1] Paper given at Histopathology Workshop.



Text-figure 1A.—Outline drawing of a vitellogenic egg chamber from a wild-type female showing the comparative sizes of the various nuclei: n, nurse nucleus; s, nucleus of squamous follicle cell; c, nucleus of columnar follicle cell. *Note* the largest of the 15 nurse nuclei are adjacent to the oocyte.

1B.—A *fes* chamber at an equivalent stage; 31 nurse cells are present. The 5 starred nuclei contained banded polytene chromosomes; b, nucleus of border cell; c, oocyte nucleus. The border cells function to form the micropylar apparatus which facilitates the entry of the sperm into the egg.

1C.—A wild-type nucleus of maximum size as seen in a section viewed with an oil-immersion objective. *Note* numerous thin chromosomes and multiple nucleoli. A single condensed mass of DNA is often seen attached to the nuclear envelope.

1D.—A section through a *fes* nucleus of maximum size. *Note* relatively few thick-banded chromosomes and single nucleolus. The vesiculate mass represents the X chromosome. The adjacent cytoplasm contains myriads of lipid droplets.

TABLE 1.—Catalogue of distribution of ring canals among 124 cystocytes found in 13 *fes* chambers: All cystocytes were nurselike except for 3 cells (*), which resembled oocytes. From unpublished data of P. A. Smith and P. Murphy.

Chamber	Total cells	Total nuclei	Total uninucleate cells with x canals				Total binucleate cells with x canals			
			1	2	3	4	1	2	3	4
1	15	20	5	2	3	0	1	3	1	0
2	12	18	5	3	1	1*	1	0	1	0
3	12	14	3	2	1	0	2	2	2	0
4	11	18	2	2	0	0	2	3	2	0
5	11	16	3	1	2	0	2	2	1	0
6	11	12	5	2	2	1*	1	0	0	0
7	8	12	3	0	0	1*	1	3	0	0
8	8	12	3	0	1	0	1	2	1	0
9	8	10	3	2	1	0	1	0	1	0
10	7	13	1	0	0	0	2	3	1	0
11	7	12	2	0	0	0	1	3	1	0
12	7	11	1	1	1	0	2	2	0	0
13	7	9	1	4	0	0	1	1	0	0
	124	177	37	19	12	3	18	24	11	0

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Proliferation and Dynamics of Cell Heredity in Blastema Cultures of *Drosophila*

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SUMMARY

Blastemas of imaginal discs isolated from *Drosophila* larvae can be continuously cultured in the abdomens of adult flies. In this medium unlimited proliferation takes place, but no differentiation into adult structures occurs. The rates of growth become specifically different in various lines and sublines. The capacities for final differentiation into adult organs are tested with fragments reimplanted into metamorphosing larval hosts. These posttests show that the initial competence required for normal development can be permanently retained, so that normotypic differentiations result even after years of culturing. Only normal karyotypes have been found in such normotypic cultures. There are no indications of any programmed aging. The following categories of changes in cell heredity occur earlier or later in proliferating cultures: 1) from autotypic to allotypic blastemas—this is the process of transdetermination; 2) from normotypic to anormotypic blastemas; 3) from telotypic to atelotypic blastemas. Some possible relations of these changes to neoplasia are discussed.—Nat Cancer Inst Monogr 31: 351–364, 1969.

THIS REPORT does not deal directly with neoplasia, but we shall see that in some long-term cultures of insect blastemas, properties of cell proliferation and capacities for specific differentiation either remain constant or change into new types of cell heredity. Such findings have a bearing on this Symposium, because in neoplasia, too, specific characteristics of proliferation are observed and the cells involved breed true for distinct and sometimes aberrant types of differentiation.

A fully grown *Drosophila* larva contains primordia, called imaginal discs, which are programmed for forming the adult body. Each disc contains a mosaic of blastemas, and the cells of each blastema are determined to give rise to specific organs or structures of the adult. In normal development, mitotic activity in the discs stops at the onset of metamorphosis. Definite differentiation sets in, and there are no more cell divisions later on. Thus, during normal development, a limited number of mitotic cycles is characteristic of these insect cells. I shall first show that such a restriction is not compulsory.

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UNLIMITED PROLIFERATION *IN VIVO*

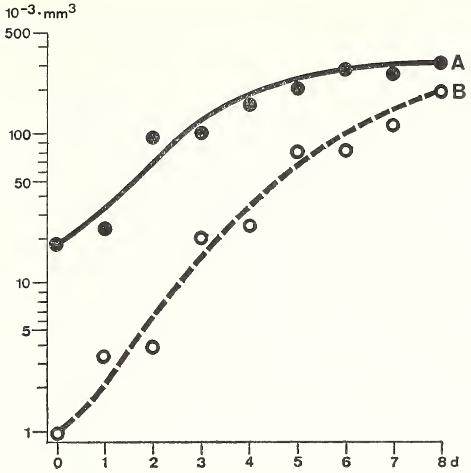
The events of metamorphosis can be circumvented by transplanting blastemas of larval discs directly into the abdomens of adult flies. In this situation cell division continues indefinitely (*1*). Meanwhile, the cells remain in their initial undifferentiated state because passing through metamorphosis is indispensable for imaginal differentiation. Our oldest cultures have now been proliferating for almost 6 years. Because of the short lifespan of the host flies, we had to transfer samples of the cultured lines every 2–3 weeks into young hosts. At the present time the 152d transfer generation is in culture. Since we can estimate the rate of growth approximately, we know that the cells of some lines accomplished several hundreds to several thousands of additional mitoses.

CHARACTERISTICS OF PROLIFERATION

Proliferation first depends on physiological properties of the host. It is greatest in fertilized females, less in virgin females, and still less in males. Proliferation further depends on specific qualities differing from cell line to cell line and propagated by cell heredity (*2*). We followed sublines whose rate of growth is about 20 times higher than that of others. Cultures with a high rate of proliferation reach asymptotically a maximal final size of about 300×10^{-3} mm^3 in about 8–10 days. Such implants fill the available space in the host's abdomen almost completely (fig. 1). The final size is independent of the initial size of an implant. Small samples of 0.5×10^{-3} mm^3 reach this size in 8 days, as do large samples of 20×10^{-3} mm^3 (text-fig. 1). Conditions in the host's hemolymph as well as the changing surface-volume ratio of the implants might act as limiting factors.

Uninjured larval discs implanted *in toto* also reach the final size typical for the prepupal stage, but hardly any additional proliferation sets in. This observation was made with the following discs: Genital, leg, eye antenna, wing, and haltere. Only when these discs become injured or fragmented will there be an excessive proliferation, resulting in blastemas that surpass normal dimensions (text-fig. 2). Growth is minimal in intact leg discs, higher in transverse halves, and still higher in longitudinal halves where the cut surface is greater than in the transverse series. The highest proliferation was found in leg disc blastemas whose cells had been dissociated, intermingled, and reaggregated (*3*). The factors that stimulate additional proliferation after wounding or dissociation are still unknown.

Another characteristic type of proliferation was observed in labial discs (*4*). Here, even an uninjured disc, which *in situ* normally forms one-half a proboscis, grows in the adult abdomen up to twice normal size, if it is cultured long enough. Thus it becomes capable of forming



TEXT-FIGURE 1.—Growth curves of a culture line with high rate of proliferation during culturing in the abdomens of new hosts. *Ordinate:* volume in 10^{-3} mm^3 (logarithmic scale). *Abscissa:* culture time in days (d). Each *circle* refers to the mean of 15 independent measurements. A: small starting size. B: large starting size.

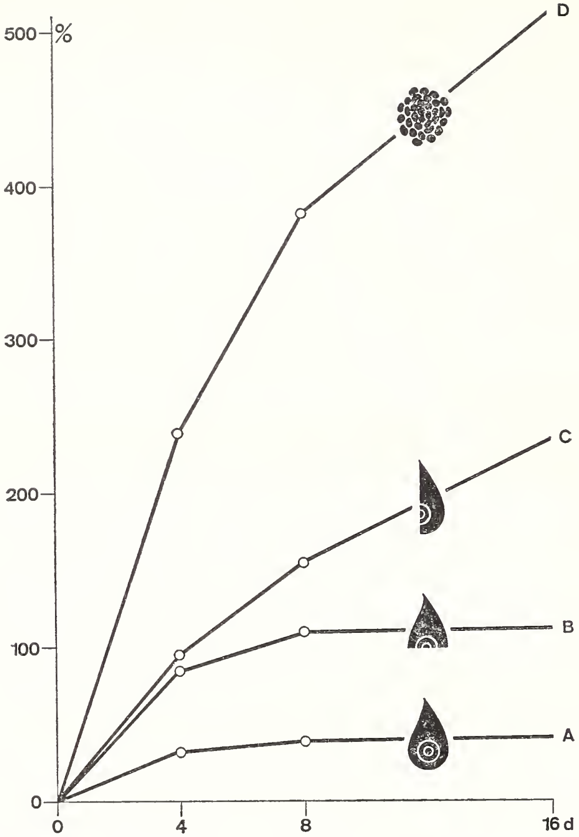
Courtesy of A. Dübendorfer, unpublished data

a complete and symmetrical proboscis when it is allowed to go through metamorphosis.

The rate of proliferation, moreover, is specifically different in various blastemas of a disc. Mindek (*5*) found a high rate of growth in blastemas of the anal plates in female genital discs, whereas neighboring blastemas of the vaginal plates and of the internal genitalia hardly proliferated. It was therefore not possible to initiate permanent cultures of the two last-named organs. We (*6*) observed a much higher rate of proliferation in distal blastemas of leg discs than in proximal blastemas of the same disc. Thus proliferation is blastema specific.

DIFFERENTIATION TEST AFTER PROLONGED CULTURING

At any desired time, samples (test implants) from our *in vivo* cultures can be reimplanted into larval hosts within which they will undergo metamorphosis [*cf.* (*1*)]. This procedure enables us to determine the degree and kind of competence for adult differentiation still present in the blastemas, which had proliferated for years and had maintained their initial character as a population of undifferentiated cells. These tests prompt the question: How will such a cell system react after hundreds of additional mitotic cycles, when exposed to the hormones that normally turn on adult differentiation?



Text-figure 2.—Growth of leg blastemas. *Ordinate:* volume of the implant in percentage of starting size of the culture in the adult abdomen. *Abscissa:* culture time in days. A: whole discs. B: transverse halves. C: longitudinal halves. D: dissociated blastemas [After Tobler, 1966 (*3*).]

NORMOTYPIC MAINTENANCE OF CELL HEREDITY

Apart from some aberrant cell lines, with which I shall deal later, we found that the test implants of many cultures differentiated into fully normal adult organs and structures. Such a normal performance is shown in figure 2. Here, parts of wings and thorax regions developed from test implants derived from transfer generation 148, after 5 years and 9 months of culturing. These structures cannot be distinguished from differentiations resulting from control implants taken directly from a larval disc. We conclude that all those genes and cytoplasmic constituents needed for normotypic differentiation can be replicated during an unlimited sequence of cellular divisions.

MAINTENANCE OF NORMAL KARYOTYPES

During growth and proliferation *in vitro*, karyotypes, as a rule, soon become abnormal. Chromosomal aberrations and also changes in the number of chromosomes lead to various types and degrees of aneuploidy. This disintegration of balanced karyotypes is an almost insurmountable handicap which has hampered conclusions concerning cellular functions and developmental potencies of the systems under study. In contrast to all these experiences, the conditions under which we culture the imaginal blastemas allow the cells to replicate their initial normal karyotypes without change. This statement is based on extensive chromosomal studies in proliferating blastemas after 2–3 years of culturing (*7*). The chromosomes shown in figure 3 are representative of hundreds of other likewise normal karyotypes. In the normotypic lines not a single aberrant mitotic figure was found. Such a normality is apparently an indispensable requirement for the observed maintenance of normotypic competence which results in adult differentiation.

NO PROGRAMMED AGING

Is aging of the soma the consequence of an inherent exhaustion or deterioration of cellular properties? Findings on continuous cell cultures have been frequently discussed with respect to this question. Hayflick (*8*) pointed to the fact that "immortal" cultures of somatic cells could not be considered as representative of nonaging normal systems. Their karyotypes as well as their morphological and physiological characteristics are so aberrant that a direct comparison with the requirements and the performance of a normal life remains most questionable. Those human and other vertebrate cells (fibroblasts) that do not enter this pathway of abnormality are, on the other hand, reported to be capable of completing only a limited number of mitotic cycles. After this performance they all die, regardless of whether they are still incorporated *in vivo* or set free in a culture *in vitro* (*8*). From this observation it was concluded that aging must be strictly programmed in the sense that somatic normality could not be permanently maintained by cell heredity. Though this might be typical for vertebrates, it certainly cannot be considered as a general "rule of life." Our normotypic blastemas do not show the slightest indication of aging or loss of their initial differentiation potentials. Normal cell heredity continues even in cultures that have now surpassed the lifespans of their donors by one hundredfold.

TRANSDETERMINATION

In all cultures that we initiated so far with blastemas of imaginal discs we witnessed qualitative changes in the specificity of cell heredity. After a certain time of culturing, parts of the test implants no longer differentiated into structures typical of the initial "autotypic" determination of the blastema. Rather, they then produced "allotypic" differentiations which corresponded to structures of body regions normally formed by blastemas of other imaginal discs. We call such a change a "transdetermination." Since the details and generalities of this phenomenon have been discussed in several recent reports (*1*, *9*, *10*) I shall allude only to a few findings which have a direct bearing on the present Symposium. Transdeterminations follow distinct sequences. For instance, if we start a culture with cells of a male genital disc, we first observe only autotypic differentiation in the test implants, such as penis, sperm pump, and anal plates. Later on, after a few transfer generations, allotypic blastemas that carry the determination for head parts or legs become established. From here, a further transdetermination usually leads to wing and finally to thorax blastemas. Such a sequence is followed by the ancestors of the test implants that differentiated eventually into the structures shown in figure 2. Any newly established state of determination can again be replicated during extended periods by cell heredity, unless further transdetermining switches occur.

There is ample evidence that transdetermination cannot be based on gene mutation. Rather, we interpret it as a change in the control systems on which the activity of region-specific genes depends. Teams of genes engaged, for instance, in head determination might become repressed and superseded by newly activated "wing genes" (*10*).

ANORMOTYPIC LINES

Besides transdetermination, which leads from one normotypic to another normotypic pathway, other changes in cell heredity were also observed. They resulted in true-breeding lines whose test pieces differentiated into aberrant structures. Examples of such "anormotypic" differentiation are shown in figures 4–6.

In one line the capacity for forming any bristles was definitely lost, whereas the differentiation of the small trichomes (hairs, etc.) that normally cover the cuticle between the bristles was unaffected by the change [(*11*) and fig. 6].

In another anormotypic line the bristles differentiated normally. Here, the trichomes of all body regions became coarse, rough, and somewhat distorted. Affected too was the arista, which also belongs to the trichome system (*12*). Test implants representing the type of differentiation before and after the change are shown in figure 5.

From abundantly proliferating normotypic blastemas carrying the determination for anal plates, no test implants which differentiated into oversized plates resulted. Rather, we observed the formation of many anal plates, all of approximately normal size and shape (fig. 6a). Apparently such excessively grown blastemas dispose of something that assures a "homonomous arealization." In one of our anormotypic lines, this particular ability for forming organs of normal dimensions, as well as the information for normal pattern formation, was lost. In this line, all the anal plate material appears to fuse into extensive bristle-bearing complexes (fig. 6b). The typical pattern is no longer recognizable, since now all bristles are of the same length and the distance between them is the same (*13*).

We interpret the changes from normotypic to anormotypic blastemas as based on somatic mutations. It is not possible to prove this assumption; however, the following facts support our interpretation: Each different type of anormotypic lines originated only once and at different times in different pedigrees after prolonged culturing. There were no intermediate forms between the normotypic ancestral and the "mutated" differentiations. Once established, the new characters were inherited without change as long as the line was cultured. A reversion to normality was never observed.

ATELOTYPIC LINES

In several cultures we observed changes leading to very fast-growing blastemas (*1*). Even the smallest implants of such lines increased in size so much that they completely filled the host's abdomen within a few days (fig. 1 and text-fig. 1). Although the longevity of the host flies can be much shortened by such cultures, the implants certainly do not play the role of malignant tumors. We never observed destruction of host organs by invasive growth. Death might result, rather, from metabolic disorders caused by the excessive proliferation of the implant.

From the following report (*14*), however, we shall learn that malignant tumors can originate from *Drosophila* blastemas cultured by our method.

The most characteristic property of our extremely fast-growing blastemas is its loss of potency for adult differentiation. Test implants of such lines never reach the final adult stage. Therefore, we call them "atelotypic" blastemas. They either disappear during the metamorphosis of their hosts or their cells remain unchanged during this process. Competence for reacting to the hormones that induce metamorphosis is definitely lost.

How are we to interpret the origin of the atelotypic lines? Again, somatic mutation might be considered. The change from a telotypic to an atelotypic blastema would then represent a lethal mutation leading to a genotype that can grow but is no longer able to differentiate any further. However, mutation can neither be proved nor excluded. Chromosomal studies did not solve the question either. Remensberger (*7*), found exclusively normal karyotypes in three of my atelotypic lines. Some of them are included in figure 3. In one line, loss or fragmentation of the Y chromosome was seen and in another line some tetraploid and octoploid (but euploid) mitotic figures were found among the normal diploid ones. In any case, atelotypic behavior can be established and propagated by cell heredity in blastemas whose karyotypes remain normal.

Phase-contrast pictures of living atelotypic cells show a most homogeneous population (fig. 7). The contact between the cells is looser than in telotypic blastemas. Moreover, basement laminae (peripodial membranes) surrounding normal discs, as well as telotypic cultures, are no longer found in atelotypic cultures (*7*).

Further morphological differences between telotypic and atelotypic blastemas have been revealed by H. Ursprung (unpublished data) who studied a few of my lines with the electron microscope. In atelotypic lines Ursprung found accumulations of virus-like particles in the cytoplasm and the nucleus. But we do not know whether the virus infection causes the atelotypic characters or whether the virus merely finds favorable conditions for multiplication in a culture that became atelotypic for some other reasons. The situation is even the more confusing, since virus particles are not completely absent from normotelotypic lines, and, in fact, are routinely seen in some other cells of the body, *e.g.*, the midgut (*15*).

Further morphological, physiological, and biochemical studies are needed before we understand better what happens when atelotypic cell heredity becomes initiated.

RELATIONS TO TUMORS

I would like to conclude this report with a few remarks about possible relations between our results and some findings on tumor cells. In both fields of investigation we are dealing with changes in cell heredity. This holds true, first with respect to proliferation. Different lines and sublines of the blastema cultures are characterized by specific rates of growth (*1*, *2*). Corresponding differences are known for tumor strains. Our transfer technique and the method of subculturing favor high rates. Such a selection is also at work when tumors first become established *in situ* and when they are cultured later *in vitro*. We found excessive proliferation, however, not only in anormotypic and atelotypic lines but also in some cultures that maintained the initial competence for normotypic differentiation. Perhaps some sort of an adaptation to the culture milieu *in vivo* might result in high growth rates.

Let us now consider the three categories of changes in cell heredity observed when test implants achieve differentiation. The first change, transdetermination, happens in determined cells during the process of proliferation. It results in blastemas capable of normal differentiation in all respects. I wonder whether any event in tumorigenesis could be based on transdetermination. This would be the case for instance, if descendants of epidermal cells, which early in embryogenesis become determined for skin formation, would differentiate into another normal cell type of different organ specificity. Teratomas are hardly convincing examples of transdetermination because we do not know whether they derive from normally determined cells or from some other and aberrant embryonic residues.

The second change from normotypic to anormotypic blastemas was interpreted as a somatic mutation. I can refer only to a controversial problem: Experts in tumor research seem still uncertain whether, and to what extent, somatic mutations might directly lead to neoplasia.

The third change initiates atelotypic blastemas. These are characterized by extremely high rates of proliferation, which are correlated with a loss of the differentiation potentials. Such a change might well find counterparts in the origin of some tumor strains. Unfortunately the methods we introduced for testing the inherent capacities for differentiation in our blastema cultures cannot be applied to cells of vertebrates and to most other organisms used in tumor research. Other difficulties for direct comparison with tumor systems arise because the blastema cultures are unique; in them, proliferation and changes in cell heredity occur in populations of "embryonic" cells whose final differentiation can be experimentally postponed or induced at will. Tumor cells, on the other hand, stand, as a rule, in the irreversible terminal phases of a developmental process.

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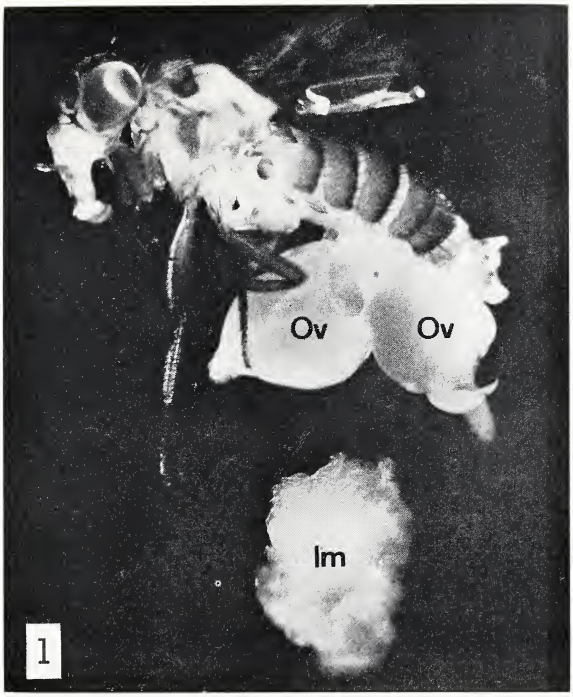


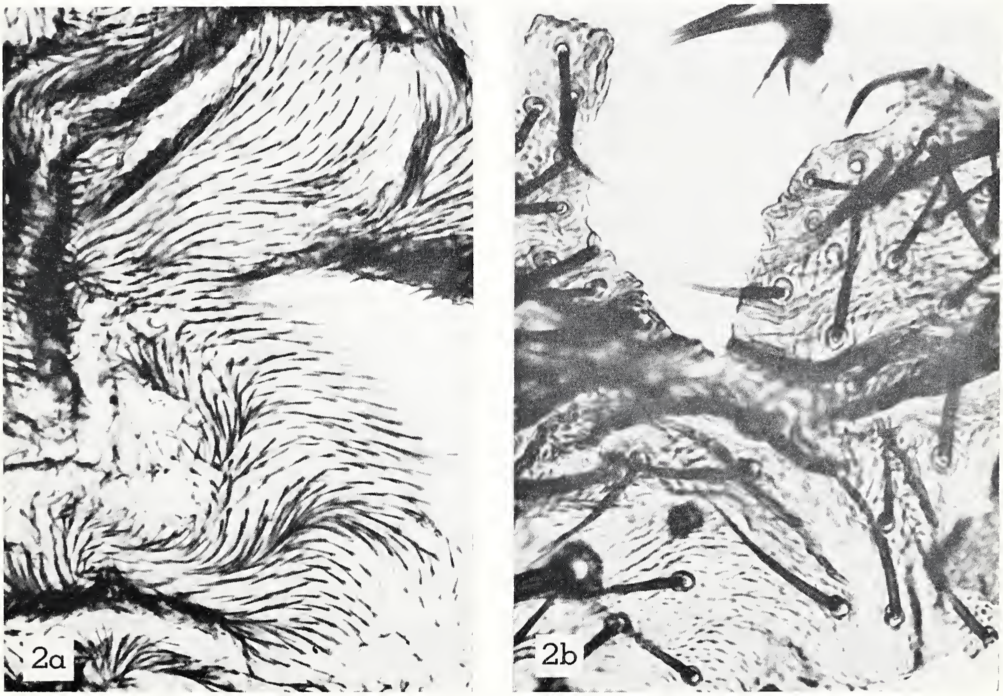
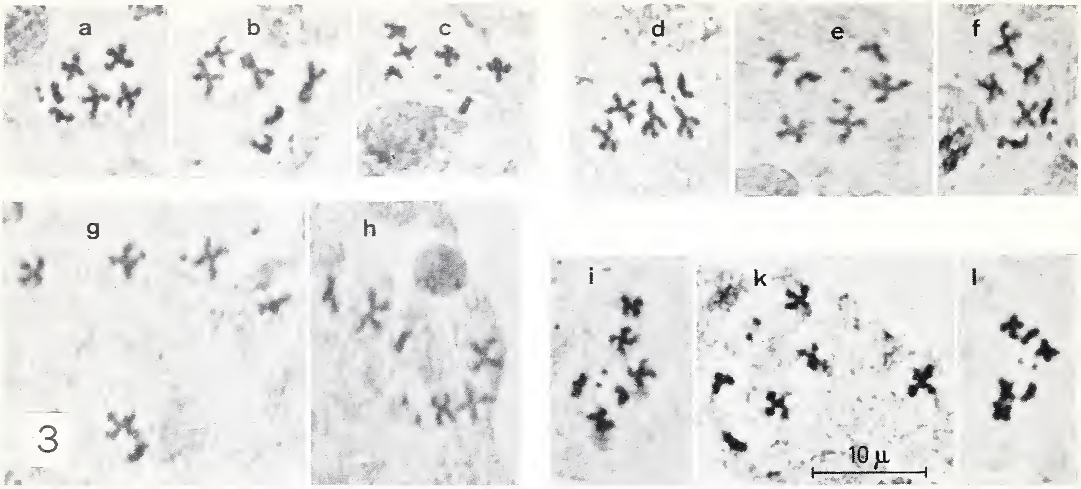


FIGURE 1.—Fast-growing implant (Im) dissected from its host after 1 week of culturing. *Top:* opened abdomen showing the full-grown ovaries (Ov).

FIGURE 2.—Parts of a wing (a) and of the dorsal mesothorax (b) formed by test implants of a permanent blastema culture.



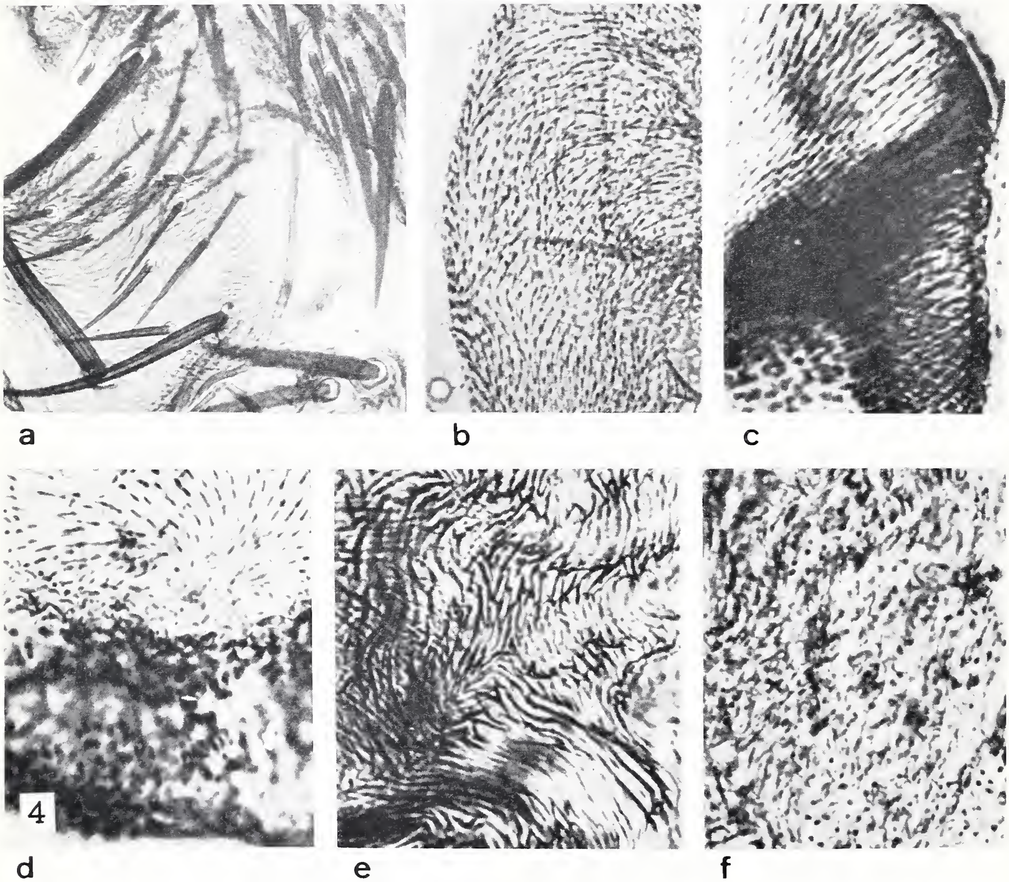


FIGURE 3.—Phase-contrast photos of normal male karyotypes in blastemas after prolonged culturing *in vivo.* a–c and i–k: from atelotypic lines; d–f: from a normotypic line; g, h: from the bristleless line.

Courtesy of *Chromosoma* (7)

FIGURE 4.—a: normotypic test implant (thorax) with bristles and trichomes. b–f: test implant of the bristleless line showing various trichome patterns on wing (b), leg (c), and different head regions (d–f).

Courtesy of *Zeitschrift für Naturforschung* (*11*)

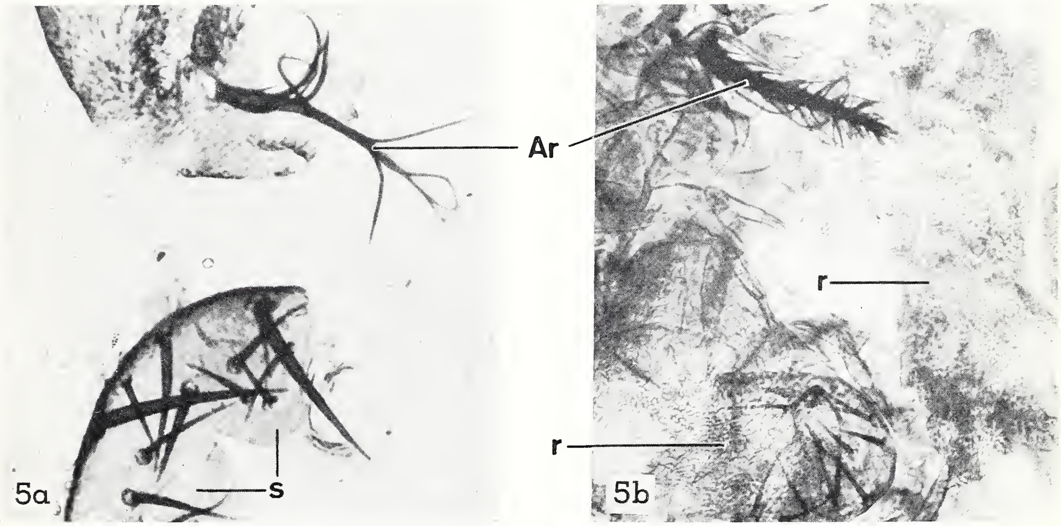
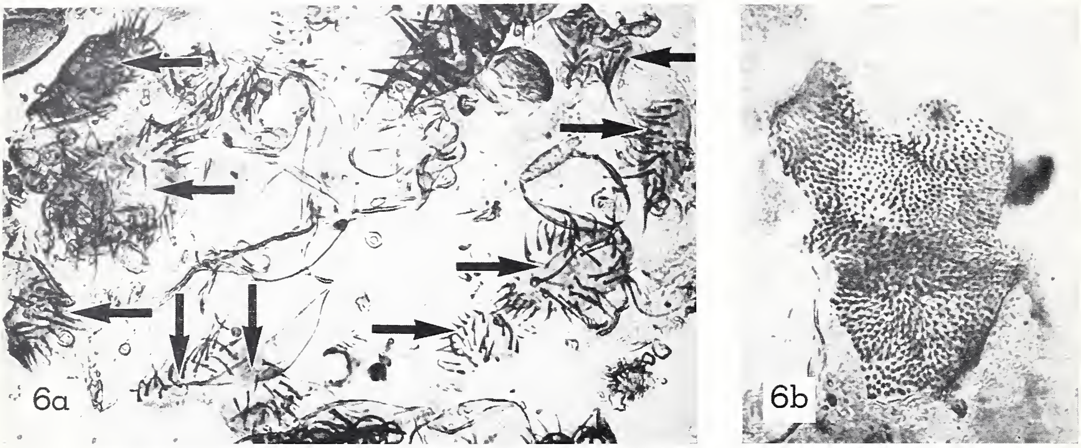


FIGURE 5.—a: normotypic head parts. b: corresponding regions of the anormotypic trichome line. Ar: Arista; s: normal trichomes on a smooth-appearing cuticle; r: rough trichomes.



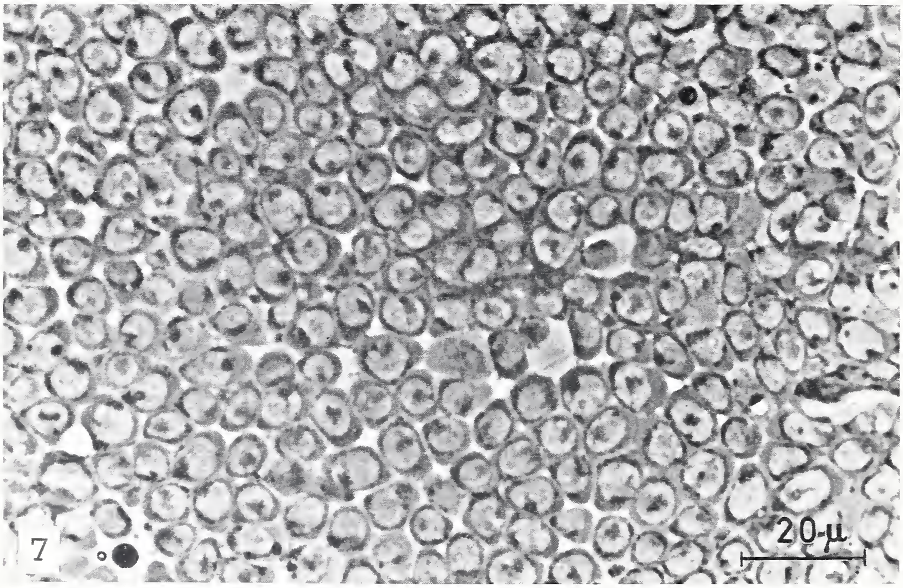


FIGURE 6.—a: arealization resulting in many normal anal plates (*arrows*) in the normotypic test implants before "mutation"; b: anormotypic anal plate.

Courtesy of *Revue Suisse de Zoologie* (*13*)

FIGURE 7.—Phase-contrast picture of an atelotypic blastema.

Courtesy of *Chromosoma* (*7*)

Neoplasms in Mutant and Cultured Wild-Type Tissues of *Drosophila* [1]

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SUMMARY

This paper describes the developmental features and structure of three neoplasms in *Drosophila melanogaster*. One of these neoplasms is an invasive neuroblastoma of the larval brain which has been transplanted from fly to fly for 45 transfer generations. It is one of the first repeatedly transplantable, invasive, and lethal neoplasms found in any invertebrate and shares many common features with malignant neoplasms in vertebrates. It is controlled by a specific recessive gene *l(2)gl*^{4} on the second chromosome. The same mutant gene is responsible for a second neoplasm found in the mutant larva, a transplantable, lethal but noninvasive neoplasm of the imaginal discs. The third neoplasm—called atelotypic tissue—spontaneously arose in cell lines derived from imaginal eye-antennal discs cultured *in vivo* in the abdomens of adult flies. Atelotypic tissue resembles in its mode of growth, developmental capacities, and structure the mutant imaginal discs and also behaves like a benign neoplasm. The developmental capacities and structure of these three neoplasms are compared with those of several other tissues of *Drosophila*, including normal brain tissue and normal imaginal discs. In addition, the general question of tumorigenesis in insects is discussed. Arguments are advanced that the infrequent occurrence of true neoplasms in insects is a consequence of certain specific peculiarities of insect development, notably the absence of cell division in most adult tissues and in many larval tissues, the destruction of many larval tissues during metamorphosis, the frequency of polyploidy, and the relative stability of the karyotype.—Nat Cancer Inst Monogr 31: 365–397, 1969.

MORE THAN a hundred papers have been published describing abnormal lesions in insects which have been called "tumors" or "neoplasms." Most of these lesions have been reported in *Drosophila* which can develop three

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[2] We thank Professor E. B. Lewis of the California Institute of Technology for mapping the new mutant *l(2)gl*^{4} and for providing balanced lethal stocks with appropriate markers. We also thank our colleagues Dr. Hiromu Akai and Dr. Lowell E. Davis for permission to reproduce their unpublished electron micrographs of *Drosophila* tissues and for helpful discussions. Dr. Peter Bryant, Dr. A. Krishnakumaran, and Mr. John Postlethwait were kind enough to criticize the typescript. Dr. J. A. Jane provided helpful discussions of neoplasms of the vertebrate nervous system. Dr. Everett Anderson provided some helpful interpretations of fine structure. Mrs. Marjorie Boyette, Miss Elizabeth Kurowski, and Mr. D. G. Osborne provided technical assistance.

types of tumors: tumorous head lesions (*1*, *2*), ovarian tumors caused by the *fs* and *fu* mutations (*3*), and a large group of so-called melanotic tumors, all of which are under genetic control and involve cells of the lymph gland, fat body, or pericardial cells (*4*). Besides these *Drosophila* tumors, about two dozen abnormal growths have been reported in other insects (*5*). However, after a detailed analysis of this literature, Harshbarger and Taylor (*6*) have concluded that none of the reported lesions in insects are comparable to the neoplasms found in vertebrates. A few of them, such as the tumorous head lesion and the *fs* and *fu* mutants, show rapid growth of specific cell types but do not lose their organ-specific characteristics (*3*). None of the tumors are lethal to their hosts. Neither are they invasive or repeatedly transplantable (*7*), as is true of many of the tumorous lesions of vertebrates. Many of the lesions appear to be little more than accumulations of blood cells at particular sites, and Harshbarger and Taylor (*6*) questioned whether insects were capable of developing true neoplasms.

In this paper we report what appears to be a true neoplasm in *Drosophila* and one of the first repeatedly transplantable lethal neoplasms found in any invertebrate. An invasive neoplasm of the larval brain, it has been transplanted from fly to fly for 45 transfer generations. It is of genetic origin and is controlled by a specific recessive gene $l(2)gl^4$ on the second chromosome. The same mutant gene is responsible for a second neoplasm found in the mutant larva, a transplantable lethal, but noninvasive, neoplasm of the imaginal discs. These two neoplasms kill the host after pupation. We shall compare the developmental capacities, histology, and fine structure of these two neoplasms with several other tissues of *Drosophila*, including wild-type brain tissue, wild-type imaginal discs, and atelotypic tissue (*8*) derived from long-term *in vivo* cultures of imaginal disc lines raised in our laboratory. In addition, we will discuss the general question of tumorigenesis in insects. Arguments will be advanced that the infrequent occurrence of true neoplasms in insects is a consequence of certain specific peculiarities of insect development. A preliminary account of this work appeared elsewhere (*9*).

MATERIAL AND METHODS

Three stocks of *Drosophila melanogaster* were used in these experiments: a wild-type Oregon R stock, mass-cultured for many years in our laboratory; a new mutant stock; and a stock of *lethal giant larva* $l(2)gl$ derived from the original Bridges stock (*10*). All stocks were raised on a standard corn meal-sugar-yeast medium at 25°C.

The new mutant gene arose spontaneously in our mass-cultured Oregon R stock. It is recessive and is located at the extreme left tip of the second chromosome at the locus 2–0.0 (*9*, *11*, *12*). Allelic to $l(2)gl$ discovered by Bridges in 1933 (*10*), it represents the fourth known mutation at this locus and has been assigned the designation $l(2)gl^4$ by usual *Drosophila* nomenclature (*10*, *12*). Throughout the paper unless indicated otherwise, the designation "mutant larva" refers to the new mutant, $l(2)gl^4$. In all experiments the ages of embryos and larvae were known to within ± 0.5 hours.

To study the capacities for growth and development of different tissues of normal and mutant larvae, we used the *in vivo* culture technique developed by Hadorn (*13*). Tissue fragments were implanted into 80-hour-old wild-type larvae and into young female adult flies. Implants into larvae enabled us to determine whether the implants responded to the various hormonal changes of metamorphosis and also their morphogenetic capacities. For example, implants into larvae *a*) metamorphose with the host, *b*) degenerate and disappear, *c*) maintain themselves but do not divide, or *d*) continue dividing and growing. Implants into adults do not metamorphose because hormonal conditions are not appropriate. They also enabled us to assess the capacity of the implants to grow, since the adult female abdomen provides a virtual living tissue culture chamber. Such implants into adults *a*) continue dividing and growing, *b*) maintain themselves but do not divide, or *c*) degenerate and disappear. If the tissue continued growing, then it was dissected from the adult after 7–14 days and cut into several pieces. Most pieces were implanted into 80-hour-old larvae to assess their capacities for metamorphosis and morphogenesis; several were reimplanted into the abdomens of new female adult hosts to continue the line. By this technique of continued subculture in adult female abdomens, cell lines could be maintained for many transfer generations over several years. In this report we will consider several cell lines derived from wild-type eye-antennal discs, wild-type embryos, and from $l(2)gl^4$ imaginal discs and brains.

For routine histologic examination, tissues were fixed in Bouin's fluid, embedded in paraffin, sectioned at 5 μ, and stained with Mayer's hematoxylin and eosin. In addition, to identify certain cell types, fragments of brain tissue were dissociated mechanically, spread in monolayers on Millipore filter discs, fixed, and stained. Autoradiographs were prepared by the procedure of Kopriwa and Leblond (*14*), as modified by Krishnakumaran *et al.* (*15*). For electron microscopic examination, the tissues were fixed in cold buffered glutaraldehyde and processed by methods described elsewhere (*16*).

RESULTS

I. General Characteristics of the New Mutant, $l(2)gl^4$

Homozygous mutant larvae do not differ in external appearance from heterozygous larvae until the beginning of the third instar, after which homozygotes and heterozygotes are readily distinguishable. During the third instar, which can be prolonged as much as 2 weeks, the fat body of $l(2)gl^4$ decreases gradually and, as a consequence, the larva becomes transparent. Moreover, the body cavity becomes distended with excess hemolymph, and old mutant larvae may become twice as long as mature wild-type larvae (fig. 1). Ultimately the larva forms an elongated puparium and dies about 3 days later without metamorphosing. Similar elongated puparia occur also in *l(2)gl* (*17*).

What underlies the peculiar appearance and developmental behavior of the mutant at pupation? When a wild-type puparium is dissected, one finds within it the growing and well-formed imaginal discs, the larval brain in the process of transforming into adult organs, and various larval tissues breaking down. In contrast, when a mutant puparium is dissected, one finds disorganized masses of mutant imaginal disc tissue and an irregularly shaped, enlarged, and asymmetrical brain. As the mutant puparium ages, the mutant disc tissue and the grotesque brain continue to grow in a disordered way but do not metamorphose and mature. The mutant disc tissue is not able to form the adult cuticle, and the mutant larval brain cannot form an adult brain. However, many of the larval tissues of the mutant continue their developmental program and begin to break down, as normally occurs during metamorphosis. Thus, in the mutant the breakdown of larval structures continues but is not paralleled by a differentiation of imaginal structures. Hence, the insect dies.

A striking feature of the mutant brain and imaginal discs is that they each give rise to transplantable neoplasms. No other mutant larval organs such as lymph gland and ventral ganglion do this. The following section describes the structure and developmental capacities of the mutant brain and the imaginal discs and the neoplasms into which they transform.

II. Structure of Wild-Type and Mutant Larval Brain *In Situ*

To understand the nature of the neoplasm into which the mutant larval brain transforms, it is necessary to compare the structures of the wild-type larval brain and the mutant brain.

Structure of the Wild-Type Larval Brain

The following description of the structure of the third instar wild-type larval brain is based on the classical study of Hertweck (*18*) and on our own observations. In the larva of *Drosophila*, all ganglia unite to form a central nervous complex located in the third thoracic and first abdominal segments. This complex consists of two brain lobes and a cone-shaped compound ventral ganglion (fig. 2). Since the ventral ganglion appears to be normal in both wild-type and mutant, the following description will be limited to the brain lobes. The brain lobes consist of a cellular cortex and an inner mass of nerve fibers, the neuropil. We will focus our attention on six major kinds of cells found in the cortex: glial cells, neurosecretory cells, neuroblasts, ganglion mother cells, preganglion cells, and larval ganglion cells. The *preganglion cells* are difficult to distinguish in histological sections of intact wild-type and mutant brains and are not designated in figure 3. However, preganglion cells can be detected easily in stained monolayers of dissociated brain cells and in *in vivo* cultures of mutant brains.

As figure 3 shows, the larval brain lobes are enveloped by a connective tissue sheath, the perineurium. The outer layer of the perineurium is noncellular. It is underlaid by a single layer of flat *glial cells*. Other glial cells are scattered throughout the brain. There are three groups of *neurosecretory cells:* a mediodorsal group (I) described by Köpf (*19*) and two other groups recently discovered by Akai *et al.* (*20*). These *cells contain*

groups recently discovered by Akai *et al.* (*20*). These cells contain neuro-

neurosecretory droplets of characteristic size and stain with neurosecretory stains. Immediately under the perineurium are the large columnar *neuroblasts* of the outer formation center which are destined to become nerve cells in the adult brain. These large neuroblasts are basophilic and their nuclei occupy most of the cell. They are the only cells in the brain in which one sees frequent mitosis. These large neuroblasts may divide equally to produce two new large neuroblasts or unequally to produce a large neuroblast and a small ganglion mother cell. The small basophilic *ganglion mother cells* are thought to divide a few more times before they differentiate into ganglion cells. They are arranged in rows and occupy the area between the outer formation center and the larval ganglion cells. The ganglion mother cells are about half the diameter of the neuroblasts. The neuropil consists of well-ordered aggregates of nerve fibers arranged in several identifiable centers. *Larval ganglion cells* are differentiated nerve cells. They are larger than ganglion mother cells, much less basophilic, and surround the neuropil and send nerve fibers into it.

Structure of the Mutant Larval Brain $l(2)gl^4$

The anatomy and cellular organization of the mutant brain differ from those of the wild-type brain. During the prolonged third instar, the brain lobes of the mutant become progressively deformed and increase to twice the size of the wild-type brain lobes (fig. 2). These gross changes in shape and size reflect striking changes in the internal structure of the mutant brain and profound differences in the spatial arrangement of the different types of brain cells (fig. 3). The six cell types occurring in the wild-type third instar larval brain are also found in the mutant brain. However, the different cell types are not localized in specific areas as they are in the wild-type brain but are intermixed with each other throughout the mutant brain. Furthermore, except for glial cells, all other cell types seem to be present in larger numbers in the mutant than in the wild-type brain.

Because of the much enlarged cellular cortex, the perineurium which envelops the mutant brain is highly stretched and vacuolated. Just as in the wild-type brain, three groups of *neurosecretory cells* are found. There appear to be more neurosecretary cells and more neurosecretory droplets per cell in the mutant brain (*20*).

As shown in figure 3, the large *neuroblasts* are found not only in the areas of the formation centers but are also scattered throughout the brain, and for that reason the formation centers are incomplete. The *ganglion mother cells* are abundant and are found everywhere in the brain intermixed with other cell types. Apparently in the mutant brain the ganglion mother cells divide many more times than in the wild-type brain and do not differentiate into neurons. The *neuropil* is often abnormal in shape and smaller than in the wild-type brain and lacks the recognizable centers of the wild-type brain. The *larval ganglion cells* are also intermixed with all other cell types but tend to remain close to the neuropil.

The increased number of brain cells, their abnormal arrangement, and their failure to mature (*see* Section III) provided us with the first hints that the mutant larval brain might be neoplastic. If this were true, then the neoplasm might be propagable by transplantation. The following experiments tested this possibility.

III. Developmental Capacities of Brain Fragments of Wild-Type and Mutant Larvae and Embryos

Brains of 96-hour-old wild-type larvae were divided into six or more fragments, and each piece was implanted into a female adult host. After 2 weeks the fragments had not grown and only occasionally could be recovered. In contrast, fragments of third instar mutant brains grew rapidly to 100 or 150 times the size of the original implant, filled the abdomens of the adult hosts with loose masses of cells which enveloped and invaded the adult tissues, and killed the host within 6 days (fig. 4). This invasive growth of mutant brain cells *in vivo* culture is unique and has never been observed in implants of wild-type brain or in cultured mutant or wild-type imaginal disc cell lines (*cf.* table 1). Not only did the cultured mutant brain tissue invade all the spaces between adult organs but it actually invaded the organs themselves, penetrating the ovarial sheath, the sheath around the midgut, and the midgut itself (*cf.* fig. 4). It may be compared with the noninvasive neoplasm in figure 5 which will be discussed in Section VI. An invasive and lethal cell line from a mutant brain has been propagated for 45 transfer generations, or more than 300 cell divisions, over a period of 9 months.

As a result of the high proliferation of the mutant brain cells, the host's abdomen appears swollen (fig. 6). We have called this peculiar syndrome "bloating." Bloating of the adult host also occurs after implantation of noninvasive neoplastic tissue (fig. 5) and will be discussed again. When fragments of wild-type or mutant larval brains were implanted into third instar wild-type larval hosts which were allowed to metamorphose, the brain fragments stopped growing. However, when fragments of mutant brain cultured for 36 transfer generations were implanted into third stage larvae, the implants continued to grow throughout metamorphosis, often killing the host before eclosion of the adult or soon thereafter. Apparently, after a period of culture *in vivo*, the mutant larval brain tissue lost its responsiveness to the hormonal changes that occur at metamorphosis (table 1).

TABLE 1.—Comparison of several neoplasms and neoplasm-like growths of different origin in *Drosophila*

Type of tissue*	Cause of abnormal growth	Rate of growth†		Mode of growth	Growth after repeated transplantation in adults	Cell types	Differentiation capacities of cells	Lethality to host	"Bloated" syndrome	Response to hormones	Type of neoplasm
		Larva	Adult								
Autotypic	—	+	+	Folded cell layers	+	One	+	0	0	+	Nonneoplastic
Allotypic	Culture	++	++	Folded cell layers	+	Two	+	+	+	+	Incipient benign neoplasm
Atelotypic	Culture and mutation	+++	+++	Compact	+	Two	0	+	+	+	Benign neoplasm
Mutant disc *in situ*	Mutation	+++	+++	Compact	+	Three	0	+	+	+	Benign neoplasm
Mutant disc in culture	Mutation and culture	+++	+++	Compact	+	One	0	+	+	+	Benign neoplasm
Mutant brain *in situ*	Mutation	+++	+++	Invasive	+	Six	0	+	+	0	Invasive neoplasm
Mutant brain in culture	Mutation and culture	+++	+++	Invasive	+	Three	0	+	+	0	Invasive neoplasm
Ten-hour wild-type embryo	Culture	?	+++	Compact	+	?	0	+	+	?	Benign neoplasm
Melanotic tumor	Mutation	+	0	Compact	0	Two	—	0	0	+	Nonneoplastic
Ovarian tumors (*fs* or *fu*)	Mutation	?	++	Compact	?‡	One	+	0	0	+	Probably benign neoplasm

*Imaginal disc cell lines which grow at a modest rate and differentiate according to their initial state of determination or prospective significance are called *autotypic* (*e.g.*, an eye-antennal disc yields cell lines which differentiate into eye-antennal structures). Imaginal disc cell lines which grow rapidly and undergo a change in determination so that they differentiate into foreign structures are called *allotypic* (*e.g.*, an eye-antennal disc cell line which differentiates into genitalia, wings, etc., in addition to eyes and antenna). Imaginal disc cell lines which grow rapidly and fail to differentiate are called *atelotypic* (8).

†+ designates normal growth rate; ++ and +++ designate increased growth rates. For details *see* text.

‡Grow after at least one transplantation (7).

These observations raised several questions about the cause of the abnormalities in the mutant brain. Are these abnormalities cell-autonomous, or are they a response of the mutant brain to an abnormal milieu in embryonic or early larval life? If mutant brain fragments are removed early enough in embryonic or larval life and placed in a wild-type environment, will they behave like wild-type or like mutant brain fragments? To answer these questions, brains were extirpated from wild-type and mutant flies at different stages of early development from 10-hour embryos to late second instar larvae. The intact brains were implanted into adult hosts. The results revealed that wild-type brains at all stages continued their growth in the adult host, finally reaching the size of a fully grown third instar larval brain. They remained compact and well formed. The host was never killed by the implant and never showed the bloating syndrome. In contrast, brains from mutant embryos and young larvae invariably grew invasively and within 8–18 days were lethal to the adult host which always became bloated. This result clearly shows that the abnormalities in the mutant brain are present before the 10-hour stage, which makes it likely that the changes in the brain cells result from their own genetic constitution and are not a response to stimuli from other tissues.

IV. Cell Types in the Transplantable Mutant Brain Neoplasm After Culture *In Vivo*

Various techniques were used to identify the cell types in the transplantable mutant brain neoplasm. In wholemount preparations of mechanically dissociated cultured brain cells monolayered on Millipore filter discs, three different kinds of cells were discernible in cell cultures of the 7th transfer generation (about 60 cell divisions) and the 15th transfer generation (about 120 cell divisions): Most cells were large neuroblasts of the formation centers and small ganglion mother cells; a few cells were elongated and looked like presumptive nerve cells, which we have termed "preganglion cells." No ganglion, glial, or neurosecretory cells were detected.

The three cell types are readily distinguishable by conventional histology and are seen in the low-power electron micrograph in figure 7. All three cell types exhibit many of the fine-structure characteristics of embryonic cells, such as scanty endoplasmic reticulum and many free ribosomes. The neuroblasts are large round cells with large nuclei. Both nucleus and cytoplasm stain deep violet with hematoxylin and eosin, and in electron micrographs the neuroblast nucleus has a higher electron opacity and more irregular shape than the other two cell types. There are numerous small mitochondria. The ganglion mother cells stain like neuroblasts with hematoxylin and eosin and are only about half the size of a neuroblast. In electron micrographs, their nuclei appear less irregular and of lower electron opacity. The elongated preganglion cells are readily identificable by their shape, size, and processes, and are often grouped together. They stain less intensely with hematoxylin and eosin than the two previous cell types. The mutant brain cells in culture attach only loosely to one another, and are readily dissociable simply by being shaken or stirred. In electron micrographs the cell surfaces appear "wavy," with occasional small cytoplasmic processes. Only here and there are close attachments seen (fig. 7, *arrows*).

Colchicine studies and autoradiographs with tritiated thymidine reveal that all three cell types synthesize DNA and divide (fig. 8). Hence all cell types contribute independently to the growth of the neoplasm. However they are developmentally related to one another: Large neuroblasts appear to be able to undergo equal division to produce two new neuroblasts or an unequal division to produce a large neuroblast and a small ganglion mother cell. The ganglion mother cells divide actively to form rows or clusters of new ganglion mother cells. They presumably give rise to preganglion cells which also divide. Whether some of these preganglion cells actually mature into nerve cells is not clear.

Thus the evidence indicates that the neoplasm is a neuroblastoma, since all three cell types found in it are neuroblasts in different developmental stages.

V. Structure of Wild-Type and Mutant Imaginal Discs *In Situ*

The nature of the neoplastic transformation which the mutant imaginal disc undergoes is best understood by a comparison of its structure and developmental capacities with those of wild-type imaginal discs. The following description of the wild-type imaginal disc is based on our own observations (*21*), and agrees in most details with the description by Ursprung and Schabtach (*22*).

The wild-type imaginal disc is a flat, folded, saclike structure which grows by cell division (fig. 9). It is enveloped by a continuous single layer of cells, the peripodial membrane. The peripodial cells are flat with a spindle-like cross section and have a thin acellular granular sheath on their outer surface (fig. 11).

The imaginal disc cells are columnar in shape and arranged in folded single-cell layers. The basal surface of the cells is attached to a basal lamina. The luminal surface contains numerous microvilli (fig. 12) and is the site of cuticle secretion when the disc metamorphoses. The apical lateral surfaces of the disc cells attach to each other by septate desmosomes (fig. 12, *inset*).

Figure 12 depicts the fine structure of wild-type imaginal disc cells. The cells have features typical of embryonic cells. The cytoplasm, which occupies only a narrow strip around the large elliptical nucleus, contains numerous free ribosomes, a moderate number of small mitochondria, and little endoplasmic reticulum. The cytoplasm of many cells contains droplets of low electron opacity. A few cells have membrane-bound vacuoles. The matrix of the nucleus consists of granular material of variable electron opacity. Within the nuclear matrix a fine granular nucleolus-chromatin complex of moderate electron opacity can be observed.

The mutant discs have a different appearance (fig. 10). Some discs are missing from their original sites, whereas in other sites discs are enlarged and may represent the fusion of several discs. The peripodial membrane often has large gaps. The mutant disc grows by cell division, just as the wild-type disc, but does not exhibit the control of size, shape, and folding pattern that characterizes the wild-type disc. Already by the middle of the second larval instar, the mutant imaginal disc has lost some of its organization and consists partly of amorphous clusters of cells loosely attached or completely detached from their original sites. By the third instar, the mutant discs have become infiltrated by undifferentiated round basophilic cells from the lymph gland (fig. 13) (*23*, *24*). The internal structure of the mutant imaginal disc is now largely lost (fig. 10). The mutant imaginal disc cells are not arranged in folded monocellular layers but form a loose amorphous mass. There are more free cell surfaces than in wild-type discs and these free surfaces are covered with cytoplasmic processes of various size and shape. No specialized junctions are found between cells. The fine structure of the mutant imaginal disc cells is also significantly different from that of wild-type disc cells (fig. 13). The familiar flat peripodial cells are no longer evident. They appear to have transformed into cells of irregular size and shape. The cytoplasm of the peripodial-like cells is more electron opaque than is that of wild-type peripodial cells. Also, unlike those of the wild-type peripodial cells, the luminal surfaces of the mutant peripodial-like cells contain numerous cytoplasmic processes. The outer surface is irregular, bounded by a layer of fine granular material which is thicker than the corresponding layer of wild-type discs. The mutant disc cells are also more electron opaque than the wild-type imaginal disc cells and vary greatly in the size and shape of both cell and nuclei (fig. 13).

VI. Developmental Capacities of Wild-Type, Mutant, and Embryonic Imaginal Discs

Not only do mutant imaginal disc cells differ in their arrangement and fine structure from wild-type imaginal cells, but they also differ in their developmental capacities.

Fragments of wild-type imaginal discs may be cultured *in vivo* in female adult abdomens for dozens of generations. Our longest cultures have been for 80 transfer generations (40 months and about 480 cell divisions), and Hadorn (*25*) has had cultures for 300 transfer generations (70 months and 1,500 cell divisions). In early culture generations, the implants grow at various rates and, over a 7- to 14-day period, may increase in size from twofold to fivefold. The host fly always survives and lives a normal lifespan.

When fragments of wild-type imaginal discs or *in vivo* cultured cell lines derived from imaginal discs are implanted into 80-hour-old larvae, they usually metamorphose with the host and produce specific integumentary structures, such as leg cuticle, wing, antenna, etc. (*8*). While retaining this capacity for metamorphosis, these lines continue to grow at a modest rate and are not lethal to the host fly. Hadorn (*8*) has called them "autotypic" (table 1).

From such metamorphosing lines, one occasionally obtains a line that does not metamorphose when implanted into a larva. Such a line can be subcultured for many generations and does not regain the capacity to metamorphose. Because this tissue cannot reach its "developmental goal," Hadron (*8*) has called it "atelotypic." Such atelotypic cell lines suddenly appeared in four of our cultures in the 8th transfer generation of a line which was started from half an eye-antennal disc of a 96-hour third instar larva. It has been subcultured for 80 transfer generations; repeatedly tested for its ability to metamorphose, it has not responded positively. In the adult abdomen this tissue grew rapidly into a compact, noninvasive mass of cells and increased in size up to 60-fold within 7–14 days (fig. 5). During this period the adults harboring the atelotypic tissue lost most of their fat body and ovaries and became distended and transparent, exhibiting the bloating syndrome (fig. 6). Finally the hosts died.

Although lethal to adults, this atelotypic tissue was not lethal to larvae. The implanted atelotypic tissue grew in larvae until the onset of metamorphosis, after which it ceased to grow, permitting the adult to develop, emerge, and live out its life. Thus the atelotypic tissue responded to the hormonal conditions at metamorphosis by ceasing further growth, but it did not differentiate into integumentary structures (table 1) (*8*).

Mutant discs behave differently from normal discs and closely resemble atelotypic tissue. When fragments of mutant discs were implanted into female adult abdomens, they grew much faster than wild-type imaginal disc cells and increased in size 30- to 60-fold to form a compact mass. The adult hosts exhibited the bloating syndrome (fig. 6) and died in about one week. Fragments derived from mutant imaginal disc tissue have been cultured *in vivo* in female adult abdomens for many generations. We have had one cell line in culture for 30 months and 50 transfer generations. This cell line continues to behave in the same way as the original mutant disc tissue and the atelotypic tissue and is lethal to the host (table 1).

When fragments of mutant discs were implanted into third instar wild-type larval hosts and allowed to metamorphose with the host, in contrast to wild-type discs, the mutant disc tissue never metamorphosed into specific integumentary structures (table 1). A similar observation was made by Grob (*26*) in his investigations of *l(2)gl*. The implanted cells grew in the larva until the onset of metamorphosis, after which they stopped dividing but did not differentiate. Cell lines derived from mutant disc behaved similarly and never differentiated into integumentary structures.

To establish how early in development the imaginal discs were affected by the mutation, mutant imaginal discs were dissected at the middle of the second instar and implanted into wild-type larval hosts of the same age. This is the earliest possible stage at which the immature disc is large enough to be handled conveniently. In all cases the young mutant disc failed to differentiate and behaved autonomously. Wild-type discs of corresponding age metamorphosed.

Further studies of the autonomy of the mutant discs were conducted with embryos and young larvae. In *Drosophila* at 25°, embryonic development lasts about 22 hours. When fragments of wild-type embryos, as early as 10 hours old, and of young larvae were implanted into wild-type adult abdomens, they gave rise to salivary glands and gut and to imaginal discs which metamorphosed into different parts of the adult integument. In contrast, corresponding fragments of mutant embryos and of young larvae gave rise to tissue which resembled mutant imaginal discs and atelotypic tissue and failed to metamorphose. This result proves that the imaginal discs of the mutant behave autonomously early in embryonic life.

Recently a cell line with lethal neoplastic properties was obtained from a fragment of a 10-hour wild-type embryo. In contrast to the wild-type implants described above, this embryonic implant gave rise to atelotypic-like tissue. In adult abdomens this tissue grew rapidly, produced a bloating syndrome, and killed the host; in larvae it was unable to metamorphose (table 1).

VII. Tissue Organization and Cell Types in Autotypic, Atelotypic, and Mutant Imaginal Disc Cell Lines

After many generations of *in vivo* culture, metamorphosing autotypic lines derived from wild-type discs resemble in most details the wild-type imaginal disc tissue described above. Peripodial cells are absent, but the cultured disc cells are arranged into saclike, folded monolayers with microvilli on their luminal surfaces and connect to each other with septate desmosomes. The most conspicuous difference between the cells of wild-type imaginal discs and autotypic tissues is in the nucleus which appears less electron opaque in autotypic tissue and has more condensed chromatin and nucleolus. Droplets of low-electron opacity present in the cytoplasm of wild-type imaginal disc cells are absent from the cells of autotypic tissue.

The atelotypic tissue looks very different. The cells are not arranged in monolayers but are packed together in large masses and do not form specialized junctional complexes. The principal cell type is highly electron opaque, has cytoplasmic processes over most of its free surface, and resembles the imaginal disc cells of the mutant (fig. 13).

The cell lines derived from mutant imaginal discs closely resemble the atelotypic cell lines. Only one cell type is present—the mutant disc cells (fig. 13). The basophilic lymph gland cells and peripodial cells, so conspicuous in the mutant disc *in situ*, are absent. The cells are packed together, form no specialized junctions between each other, and their fine structure does not differ from that of the atelotypic tissue described above even though their origin is completely different.

VIII. Comparison Between the Structure and Developmental Capacities of $l(2)gl^4$ and $l(2)gl$

The new mutant $l(2)gl^4$ is allelic to *lethal giant larva* $l(2)gl$. Heterozygous larvae of the genetic constitution $l(2)gl^4/l(2)gl$ are larger than homozygotes $l(2)gl^4/l(2)gl^4$ or $l(2)gl/l(2)gl$, and the mutations are probably different. So far as we can judge, all the results reported above with $l(2)gl^4$ also hold true for $l(2)gl$. In the following discussion, all the conclusions concerning $l(2)gl^4$ can probably be extended to $l(2)gl$.

DISCUSSION

Operational Definitions of Neoplasms in Vertebrates and Insects

The diagnostic features of neoplasms and their terminology derive almost exclusively from vertebrate pathology and oncology. The word "neoplasm" denotes a "new growth." When qualified as malignant, it defines a rapidly proliferating new growth which is invasive, infiltrating, or metastatic. In contrast, a "benign neoplasm" denotes a rapidly proliferating new growth which is not invasive, infiltrating, or metastatic. The term "tumor" is less explicit and is applied to various swellings of diverse origin (*27*).

Since *normal* growth and development in both vertebrates and invertebrates have numerous homologies, then *a priori* we might also expect *abnormal* growth in both groups to share many homologies. However, vertebrates separated from other major animal groups more than 500 million years ago and have had extensive opportunities for evolutionary innovation. Moreover they possess certain developmental peculiarities which have not yet been found in invertebrates and which might have made many of their abnormal growths different from those of invertebrates. For example, a conspicuous peculiarity of vertebrates is the possession of an immunological response system which produces circulating antibodies in response to foreign proteins. Such systems have not been demonstrated in invertebrates. Hence the possibility existed that neoplastic lesions in vertebrates and invertebrates would not share homologies.

In this discussion, arguments will be presented that extensive homologies exist between vertebrate neoplasms and the two neoplasms of genetic origin described in this paper. Evidence will be offered that the mutant brain tumor shares many features with malignant neoplasms of vertebrates and that the mutant imaginal discs share features with benign neoplasms of vertebrates. Similarities between neoplastic transformation of vertebrate cells *in vitro* and the atelotypic transformation of imaginal disc cells cultured *in vivo* will also be considered. Finally, reasons will be sought to explain the low incidence of neoplasms in insects.

Similarities Between the Mutant Brain and a Malignant Neoplasm

Let us first consider the behavior of the mutant larval brain *in situ.* In the mature mutant larva, the three kinds of neuroblasts present in the brain—large neuroblasts, ganglion mother cells, and preganglion cells—cease to grow in an ordered way. Not only do they grow more rapidly than wild-type neuroblasts, but they invade areas normally foreign to them so that the normal pattern of the brain is drastically disturbed (*cf.* fig. 3). Moreover the rapid and invasive growth of neuroblasts does not terminate with differentiation into ganglion cells. In the mutant, the final differentiative step to ganglion cells is blocked and the neuroblasts continue dividing.

Experiments in which fragments of mutant brain were implanted into adult abdomens emphasize both the invasiveness, rapid growth, and failure of the neuroblasts to differentiate into nerve cells. In the adult abdomen, unhindered by the perineurium, the mutant neuroblasts invade the body of the host, envelop and penetrate its organs, destroy its fat body and ovaries, and finally kill it (fig. 4). Another striking feature of the invading neuroblasts in an adult abdomen is their low adhesiveness and ready dissociability, contrasting sharply with normal brain cells which do not dissociate so easily (fig. 7).

We thus see several features which the mutant brain tissues share with malignant neoplasms of vertebrates: 1) rapid growth both *in situ* and after culture *in vivo*, 2) repeated transplantability, 3) invasiveness both *in situ* and after transplantation, 4) low adhesiveness of the cells to one another, and 5) lethality to the host (table 1). If one were to list the diagnostic features of a malignant neuroblastoma in an insect using vertebrate malignant neuroblastomas as a model (*28*, *29*), then the several characteristics of the mutant just enumerated would head the list. For this reason, it seems fair to argue that the mutant brain has become a transplantable malignant neuroblastoma. So far as we are aware, it is only the second brain tumor reported in insects (*30*) and the second genetically controlled malignant neuroblastoma in animals (*31*).

The results of the transplantation experiments with brains of embryos and young larvae indicate that the genetic defect that transforms the brain of the homozygous mutant larva acts directly on the neuroblasts which are destined to form the adult centers of the brain and not indirectly via the mutant embryonic or larval milieu. Similarly, the mutation has little effect on the purely larval elements of the brain which look normal and apparently function normally since the larva feeds and behaves normally until the onset of adult development.

The changed developmental capacities of these mutant neuroblasts are accompanied by ultrastructural changes, the most striking of which is the presence of a "wavy" surface. Whether the "wavy" surface is causally connected with the low adhesiveness of the mutant neuroblasts is unknown. Another ultrastructural feature of the mutant brain is an increase in the number of neurosecretory cells and of neurosecretory droplets within the cells when compared to a wild-type brain. The significance of this observation is uncertain.

Similarities Between Mutant Imaginal Discs and Benign Neoplasms

The mutant imaginal disc tissues *in situ* behave differently from wild-type discs. They grow at different rates and no longer form the neatly folded monolayers of cells that characterize the wild-type discs. Instead, the cells form amorphous clusters of variable size and shape. Moreover, they are unable to differentiate into chitinous integumentary structures even when implanted into wild-type larvae. The results of culturing mutant discs *in vivo* in adult abdomens emphasize both the rapid growth and the loss of a normal pattern of growth. They also underline the fact that the growth is not invasive like that of the mutant brain (fig. 5). However, like the mutant brain tissue, the mutant disc tissue invariably kills the adult host and, in this respect, is wholly different from wild-type disc tissue or autotypic tissue (table 1).

Fragments of mutant discs from young larvae also failed to metamorphose when implanted into wild-type larvae, indicating that the mutant disc tissue is affected directly by the mutation.

The mutant disc cells thus exhibit rapid growth both *in situ* and after transplantation, loss of ability to differentiate normally, loss of normal pattern of growth, ability to be propagated by transplantation, low adhesiveness, and lethality to the host. They are not invasive. These features are shared with many benign neoplasms of vertebrates and support the view that the mutation has transformed the imaginal disc into a benign neoplasm (*cf.* table 1).

The ultrastructural differences between mutant and wild-type discs also demand comment. The most remarkable difference is in the cell surface. In wild-type disc cells, microvilli are found only on one surface of the cell and are associated with cuticle secretion (*21*, *22*). Furthermore, the cells are coupled to each other by septate desmosomes (fig. 12). In the mutant disc, however, cytoplasmic processes are found all over the free cell surface and probably have no role in cuticle secretion, since mutant disc cells do not make cuticle (*9*, *26*). Also, no specialized junctional complexes were observed. These cytoplasmic processes which seem to prevent the disc cells from "getting close to one another" and the absence of tight junctional connections between the cells may explain why mutant discs cannot form a normal pattern and also why they never differentiate. Another noteworthy difference between mutant and wild-type discs is the more electron-opaque nucleus and the high concentration of ribosomes in the mutant disc cytoplasm.

It remains unclear whether the cells enveloping the mutant disc are modified peripodial cells or disc cells. The invasion of the mutant disc by cells from the enlarged lymph gland is also striking and suggests that the abnormally growing mutant tissues may stimulate an intense hematopoietic reaction such as occurs in mutants developing melanotic tumors (*4*).

Atelotypic Tissue as a Benign Neoplasm

Of considerable interest is that the atelotypic tissue derived from an eye-antennal disc closely resembles cultured mutant disc tissue in both structure and developmental capacities. Like cultured mutant disc cells, the atelotypic cells have a high electron opacity, do not possess specialized junctions, and have cytoplasmic processes over their free surfaces. They grow rapidly, form an amorphous mass of tissue, do not differentiate, are readily and repeatedly transplantable, have low adhesiveness, and are not invasive. Thus the atelotypic tissue also behaves like a benign neoplasm (table 1). In the atelotypic tissue, the neoplastic transformation occurred spontaneously in about the 8th generation of culture. It is a stable transformation and has been cultured for 80 transfer generations. In $l(2)gl^4$, the mutation causes a similar neoplastic transformation in the imaginal discs and mutant disc tissue, has a stable phenotype, and has been cultured *in vivo* for 50 transfer generations. Thus the mutation and the atelotypic transformation provoke similar developmental and phenotypic characteristics. Apparently, the $l(2)gl^4$ mutation converts imaginal disc tissue into tissue with atelotypic characteristics.

The cause of the spontaneous atelotypic transformation in cultured discs is unknown, but it is probably the result of a somatic mutation or a larger change in karyotype (*32*). It remains to be seen whether other genetic mutations and spontaneous atelotypic transformations which cause imaginal disc cells to lose their capacity to differentiate exhibit similar morphological and structural changes and produce benign neoplasms.

Virus-like particles in several tissues of *Drosophila* have recently been described independently by several workers (*16*, *33*, *34*), and they seem to be present in considerable amounts in atelotypic tissue (*16*, *21*). This fact taken by itself might suggest that these virus-like particles caused the atelotypic transformation, perhaps in the same way that monkey virus SV40 transforms normal human cells into cancer cells. However, it is unlikely that the virus-like particles caused the atelotypic change, because a similar change can be produced by the $l(2)gl^4$ mutation. More probably these virus-like particles are more common in rapidly growing cells of all sorts; hence they occur in atelotypic tissue, mutant brain, and imaginal discs (*16*).

Effects of Hormones on Metamorphosis and Growth of Discs, Brains, and Atelotypic Tissue

A final matter which deserves comment is the response of various tissues to the hormonal changes at metamorphosis (summarized in table 1). Although the endocrinology of the Diptera is not fully understood, the main endocrine events at metamorphosis are thought to involve the release of a molting hormone—presumably one or more of the ecdysones—and a decrease in the concentration of juvenile hormone. Wild-type imaginal discs and cell lines derived from them respond to hormonal changes at metamorphosis by ceasing to divide and to grow and by secreting cuticular integumentary structures. Mutant discs, cell lines derived from them, and the atelotypic lines described in this report failed to secrete a cuticle at metaphorphosis but nonetheless stopped dividing and growing. Mutant brain tissue similarly failed to differentiate at metamorphosis and also stopped dividing and growing (table 1). Thus mutant tissue and atelotypic tissue respond to hormonal changes at metamorphosis, not by differentiating into adult structures but by ceasing to grow. Thus metamorphosis effectively rids the insect of implanted larval neoplasms, and normal adult development and adult life ensue. The only tissue we have studied which ignores the hormonal signals at metamorphosis is the cell line derived from *in vivo* cultures of mutant brains; this cell line continues dividing before and after metamorphosis of the host.

It should also be mentioned that, in addition to provoking neoplastic changes in the brain and the imaginal discs, the *l(2)gl* mutation has profound effects on the structure (*35*) and function (*17*) of the prothoracic gland cells of the ring gland and on the salivary glands (*36*).

Some Basic Differences Between Vertebrates and Insects That May Influence the Frequency of Neoplasms

The evidence presented in this report and discussed above establishes that insects have both invasive (malignant) neoplasms and noninvasive (benign) neoplasms. These neoplasms appear to be uncommon: So far as we are aware, the invasive brain neoplasm is the first transplantable malignant neoplasm recorded for an insect, and the mutant imaginal disc tissue and the atelotypic disc tissue are among a very few transplantable benign neoplasms found in insects. The remarkable ovarian tumors studied by King and his associates might be repeatedly transplantable in adults, but this has not yet been done (*7*).

Why are neoplasms uncommon in insects? The scarcity of neoplasms in insects when compared with vertebrates may be due in part to lack of detailed study. However it may stem from certain basic differences between vertebrates and insects that influence the frequency of tumorigenesis. These basic differences include the following:

a) In adult verterbrates almost all cell types divide and neoplasms are common. In most adult insects that have been studied, Lepidoptera and Diptera for example, virtually no cells in the adult divide except for those in reproductive organs and some blood cells (*15*, *37*). Because so little cell division normally goes on, an adult insect is not a likely place to find a neoplasm.

b) Most insects depend on metamorphosis. During metamorphosis many larval cells die, and much of the adult organism is constructed from imaginal disc cells and histoblasts which were never a functional part of the larval organism. Because of this reliance on imaginal discs, many injuries and defects in larvae have no effects on adults. This practice of destroying many normal larval tissues at metamorphosis probably extends to abnormal larval tissues as well. One might expect most larval tumors to disappear at metamorphosis.

c) Polyploidy and polyteny occur freqently in insects. In Diptera for example, most larval epidermis and most other larval cells as well grow without cell division. The cells enlarge and become polytene or polyploid but do not divide. Because they do not divide, even a potentially neoplastic stem cell could not give rise to a neoplasm.

d) If some tumorigenesis depends on somatic mutations, the multiple copies of genes in the polytene larval cells of Diptera and the polyploid larval cells of other insects make it unlikely that a somatic mutation in such a larval cell will express itself.

e) In vertebrates, cells cultured *in vitro* rather promptly become aneuploid or develop some other karyological defects (*38*). In insects, however, we have kept a number of cell lines in *dauer* (continuous) culture *in vivo* for nearly 4 years and more than 480 cell divisions and have detected no obvious chromosomal abnormalities. In addition, Hadorn (*25*) has kept some lines for more than 6 years and more than 1,500 cell divisions and he has not detected obvious chromosomal abnormalities (*32*).

f) There is also other evidence that the karyotype and the genetic program of insect cells are singularly stable when compared with vertebrate cells. In vertebrates, normal euploid cells, either in culture or implanted into a living animal, appear to be capable of only a limited number of divisions—about 50 in the case of human fibroblasts. After this the cells cease dividing (*39*, *40*). This is not true in insects where imaginal disc cells that have been kept in *dauer* culture *in vivo* for more than 1,200 generations are still going strong and are capable of differentiating into normal adult structures (*25*). Apparently when insect cells replicate their DNA, they do not accumulate the copying errors that are thought to place a limit on the life of vertebrate cells. Perhaps insects have efficient error-repairing mechanisms for their DNA, which would certainly reduce tumorigenesis.

Another factor which may account in part for the scarcity of neoplasms, particularly invasive neoplasms, is the absence of a capillary system in insects which penetrates extensively into all organs and tissues and provides a route for the dispersal of neoplastic cells. On the other hand, excepting the nervous system, most insect organs consist of only one or two layers of cells and are in direct contact with the blood.

These reasons probably account in part for the rarity of neoplasms in insects. However, when neoplasms do occur, where would one except to find them in a fly, for example? The arguments presented above indicate a likely site is in a larva in cells that normally divide. This excludes most larval cells but includes imaginal disc cells, neuroblasts, gonads, and blood cells, *i.e.*, the places where neoplasms have been found. In the adult, neoplasms would be expected only in the reproductive cells, as in the *fs* and *fu* mutants studied by King and his group (*3*). One would also expect to find neoplasms in embryos where there is much cell division. None of the many genetically controlled embryonic lethals of *Drosophila* have been studied with this in mind, but it seems likely that some of them will turn out to produce embryonic neoplasms. Indeed, we have recently confirmed this prediction.

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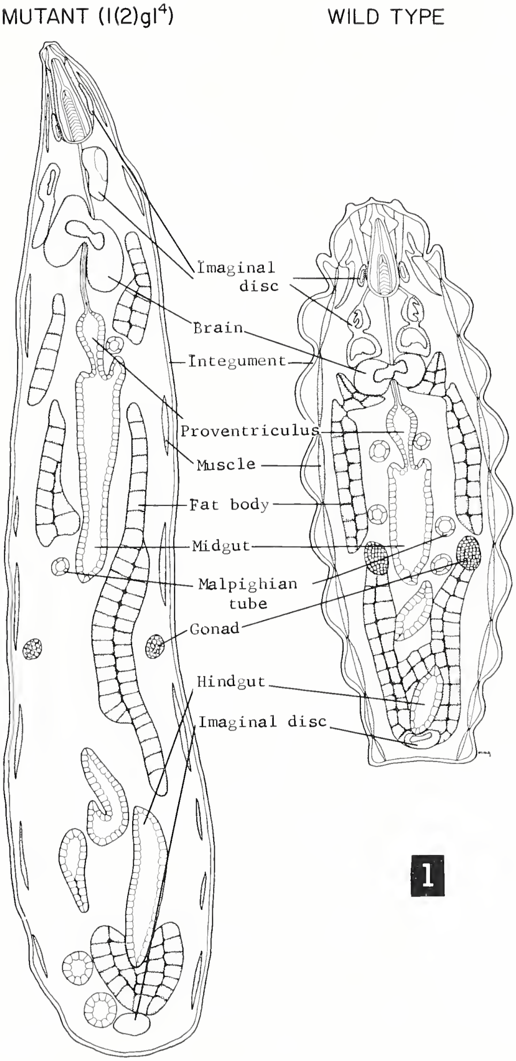

MUTANT (l(2)gl⁴)
WILD TYPE
Imaginal disc
Brain
Integument
Proventriculus
Muscle
Fat body
Midgut
Malpighian tube
Gonad
Hindgut
Imaginal disc
1

FIGURE 1.—Frontal section through third instar mutant [$l(2)gl^4$] and wild-type larvae. × 20

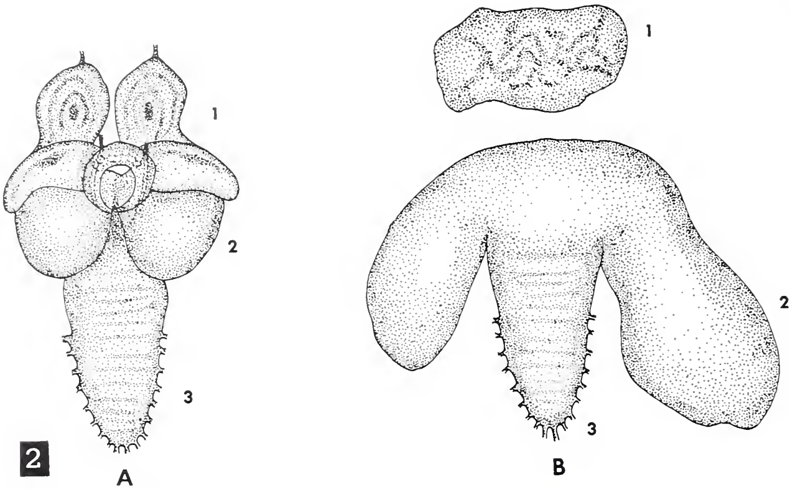


Figure 2.—Cephalic complex of: *A*, third instar wild-type larva; *B*, mutant larva. *1*, eye-antennal disc; *2*, brain; *3*, ventral ganglion. × 215

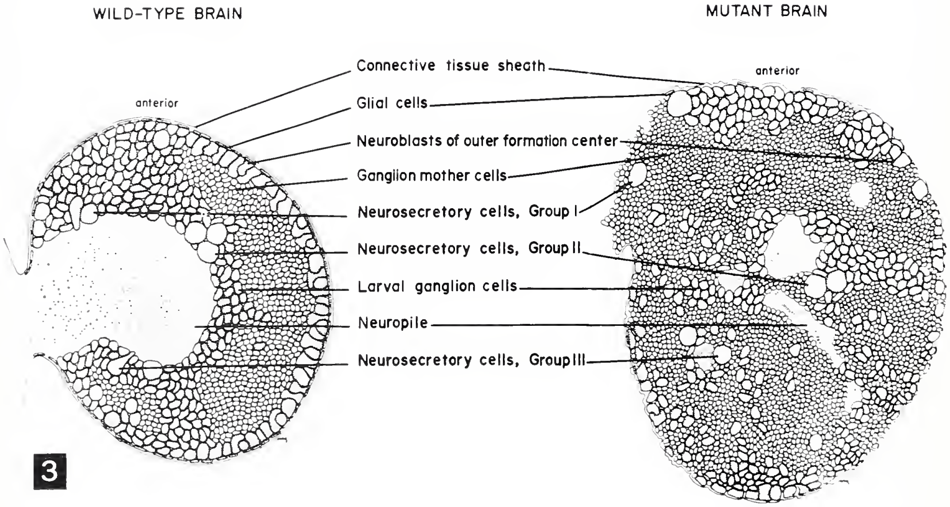
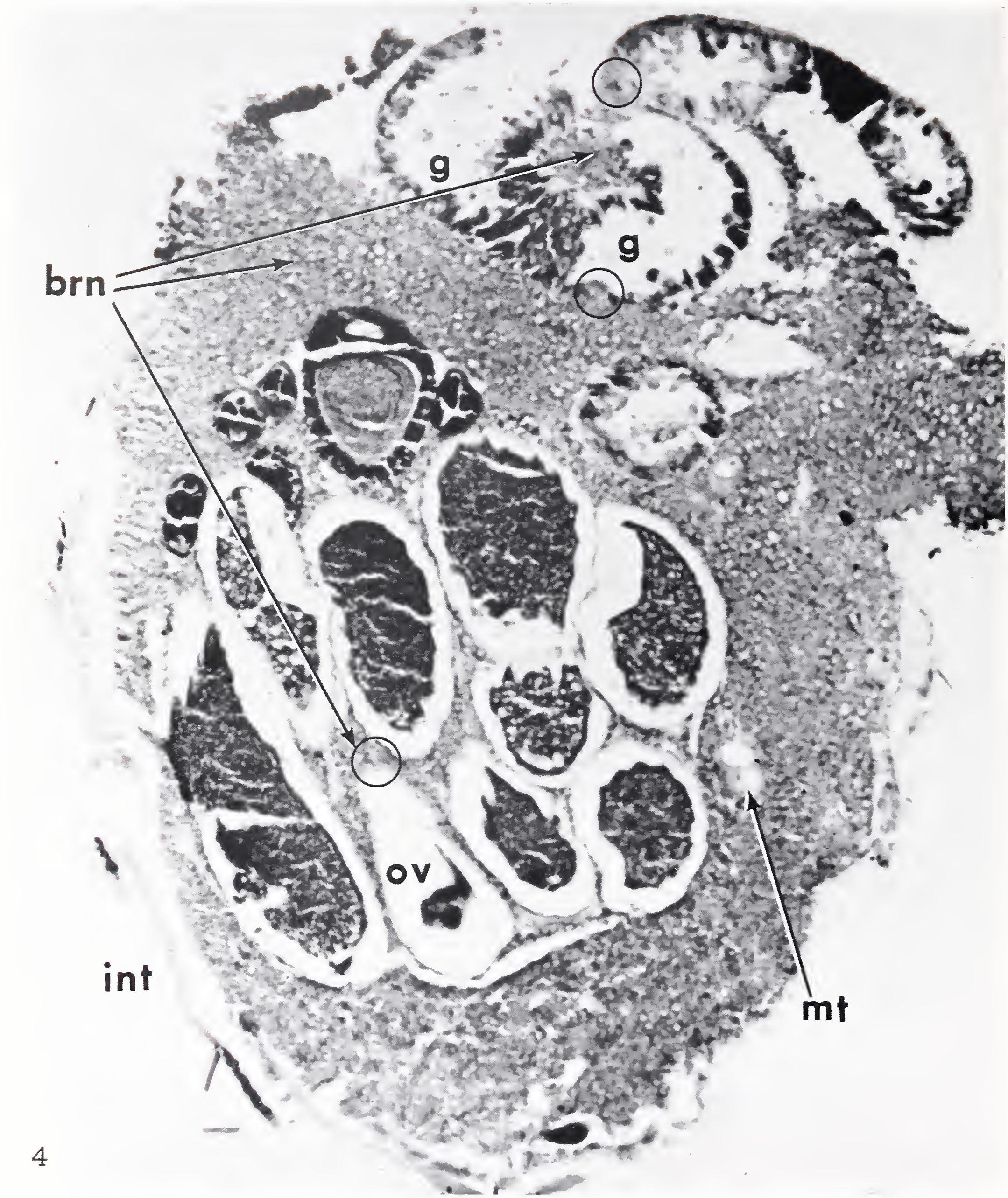

WILD-TYPE BRAIN
MUTANT BRAIN
anterior
anterior
Connective tissue sheath
Glial cells
Neuroblasts of outer formation center
Ganglion mother cells
Neurosecretory cells, Group I
Neurosecretory cells, Group II
Larval ganglion cells
Neuropile
Neurosecretory cells, Group III
3

Figure 3.—Frontal section through third instar wild-type and mutant brain (schematic). × 280

PLATES



4

FIGURE 4.—Cross section through abdomen of adult host bearing transplantable neuroblastoma from $l(2)gl^4$. *brn*, brain neuroblastoma; *ov*, ovaries; *int*, integument; *g*, gut; and *mt*, malpighian tubules. *Note* way in which the neoplasm envelops *ov*, penetrates the ovarial sheath, and invades *g* (*circles*). Fat body has already been destroyed. Toluidine blue. × 70

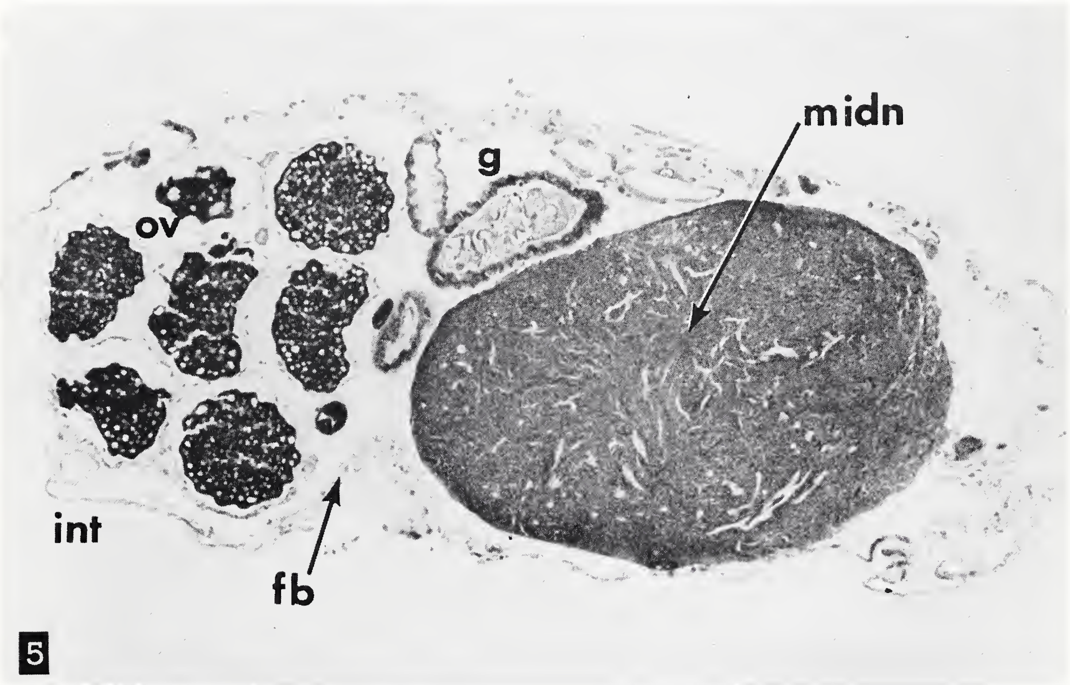


FIGURE 5.—Cross section through abdomen of adult host bearing noninvasive neoplasm of mutant imaginal discs. *midn*, mutant imaginal disc neoplasm; *ov*, ovaries; *int*, integument; *g*, gut; and *fb*, fat body. *Note* compact growth of neoplasm and reduced *fb* and *ov*. Hematoxylin and eosin. × 70

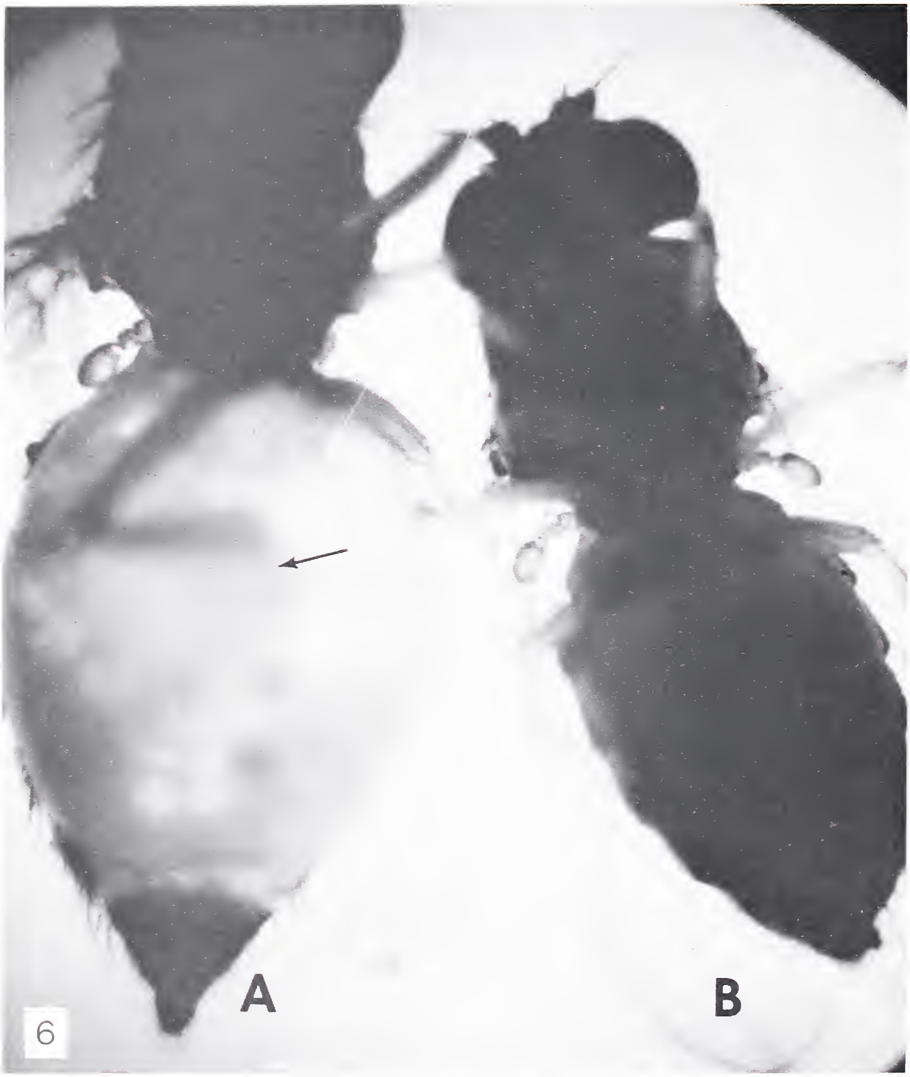


FIGURE 6.—Female adult host bearing benign neoplasm of *$l(2)gl^4$* imaginal disc. Fly *A* shows "bloating" syndrome. Fly *B* is an uninjected control. *Arrow* indicates location of neoplasm. Fat body and ovaries are almost completely destroyed, and abdomen appears translucent and swollen, when the host bears a benign neoplasm. × 40

FIGURE 7.—Electron micrograph of cultured mutant brain neoplasm from the 7th transfer generation. *nbl*, neuroblast: *gm*, ganglion mother cell: *pg*, preganglion cell: and *cp*, cytoplasmic process (*arrow*). *Note* lack of extensive contacts between cells, "wavy" cell surface (*circles*), and difference in electron opacity between nuclei of 3 cell types. Cells attach to each other in several places (*arrows*). Uranyl acetate and lead citrate. × 5700

Courtesy of Dr. H. Akai and Dr. L. E. Davis

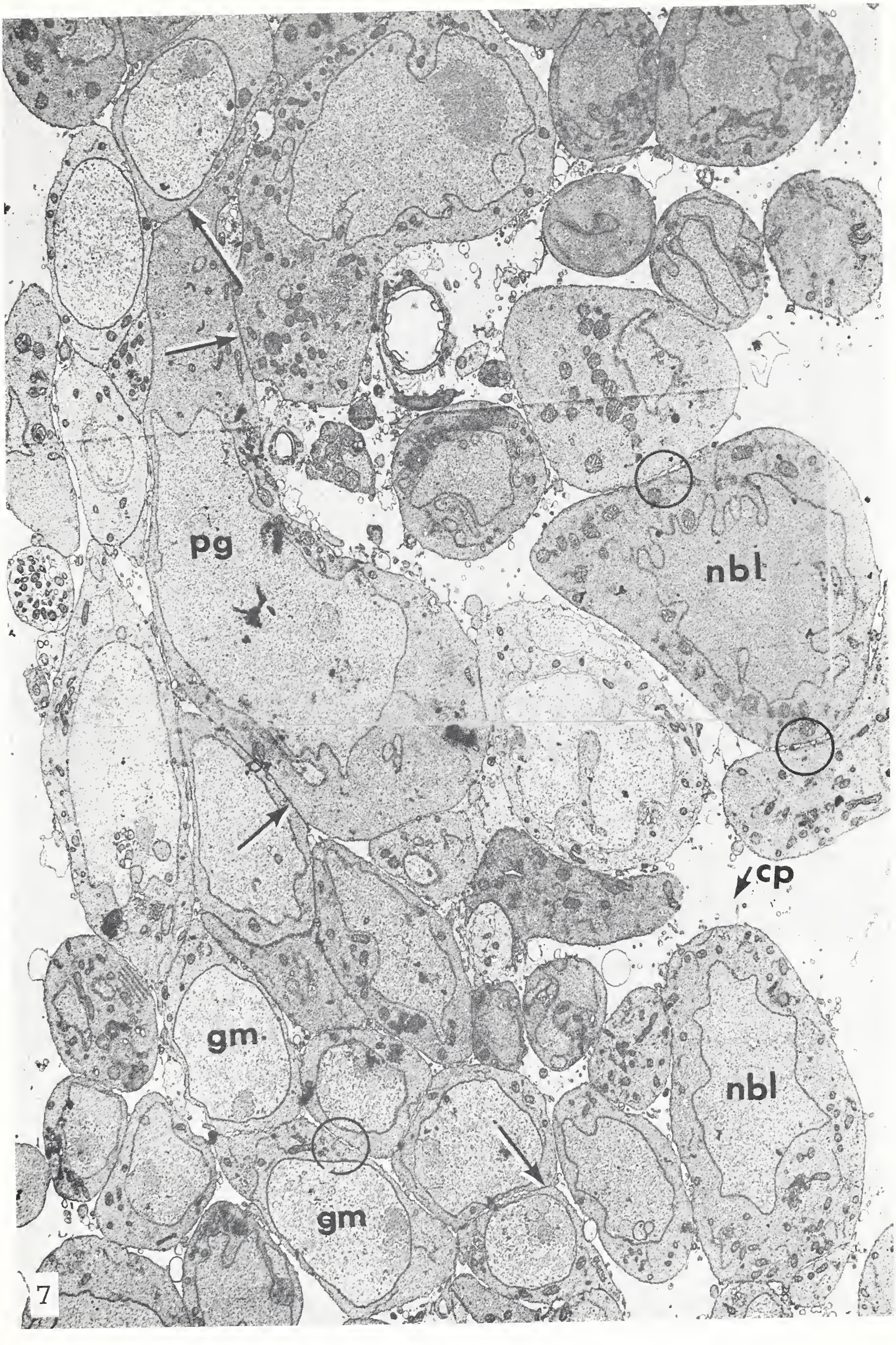
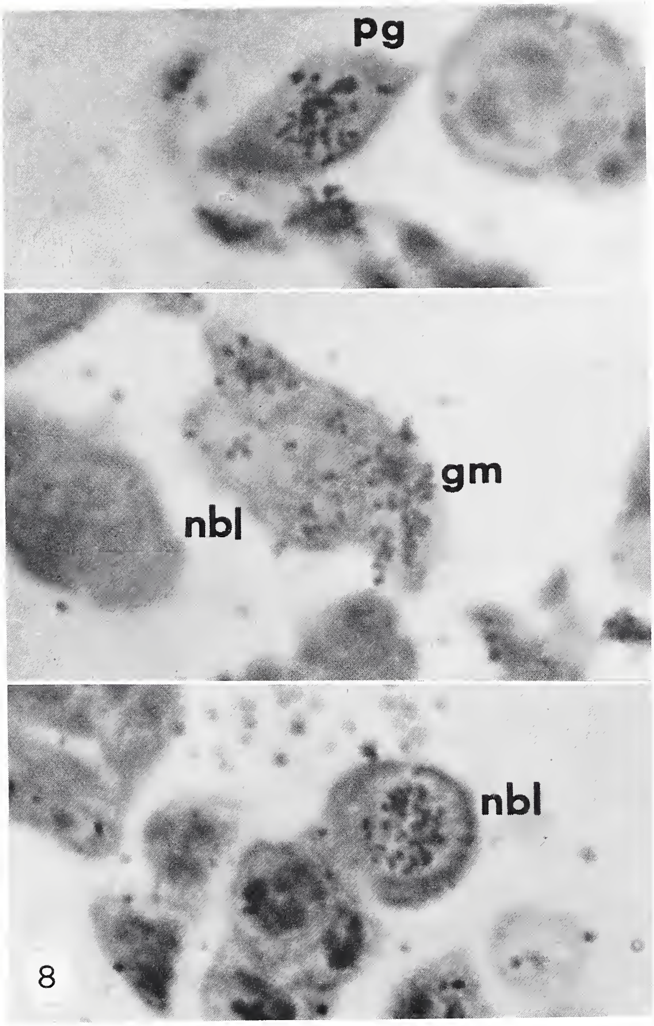
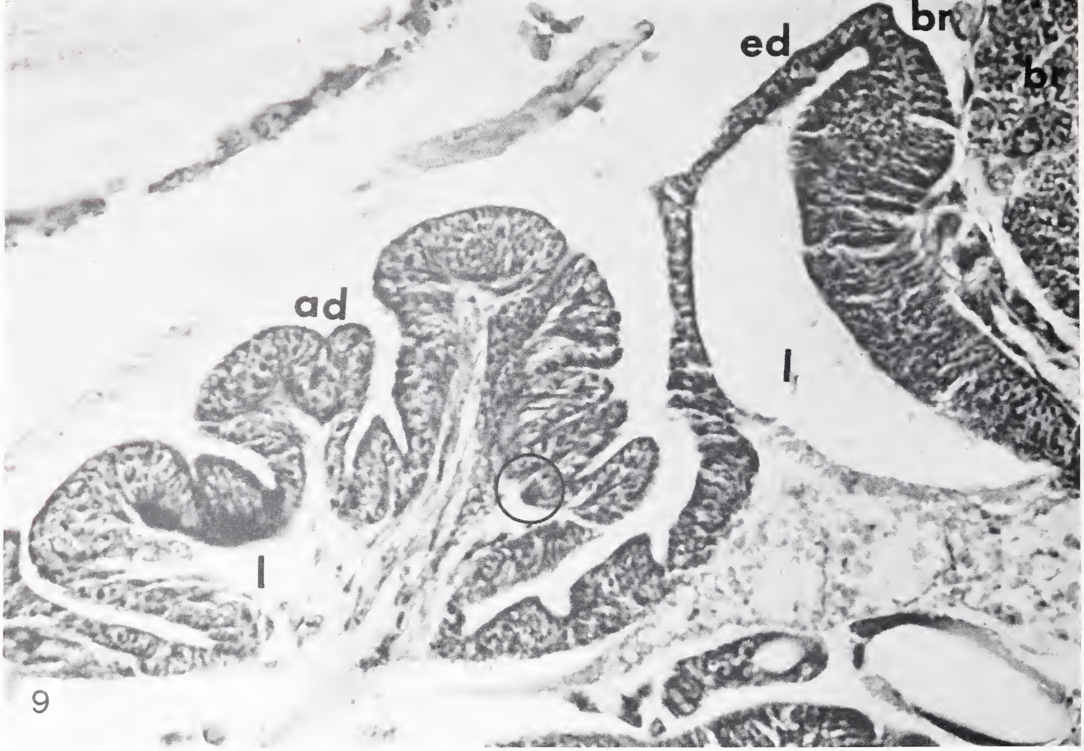

pg
nbl
cp
gm
gm
nbl
7

FIGURE 8.—Autoradiograph of cells from mutant brain neoplasm from 7th transfer generation. *nbl*, neuroblast; *gm*, ganglion mother cell; and *pg*, preganglion cell. Nuclei of all 3 cell types are intensely labeled. Neoplasm-bearing animals were given injections of ^{3}H-thymidine (10 μc/g live weight for 90 min); the cells were dissociated mechanically and then were spread on Millipore filter discs, fixed, and processed for autoradiography. Hematoxylin and eosin. × 1330


pg
gm
nbl
nbl
8



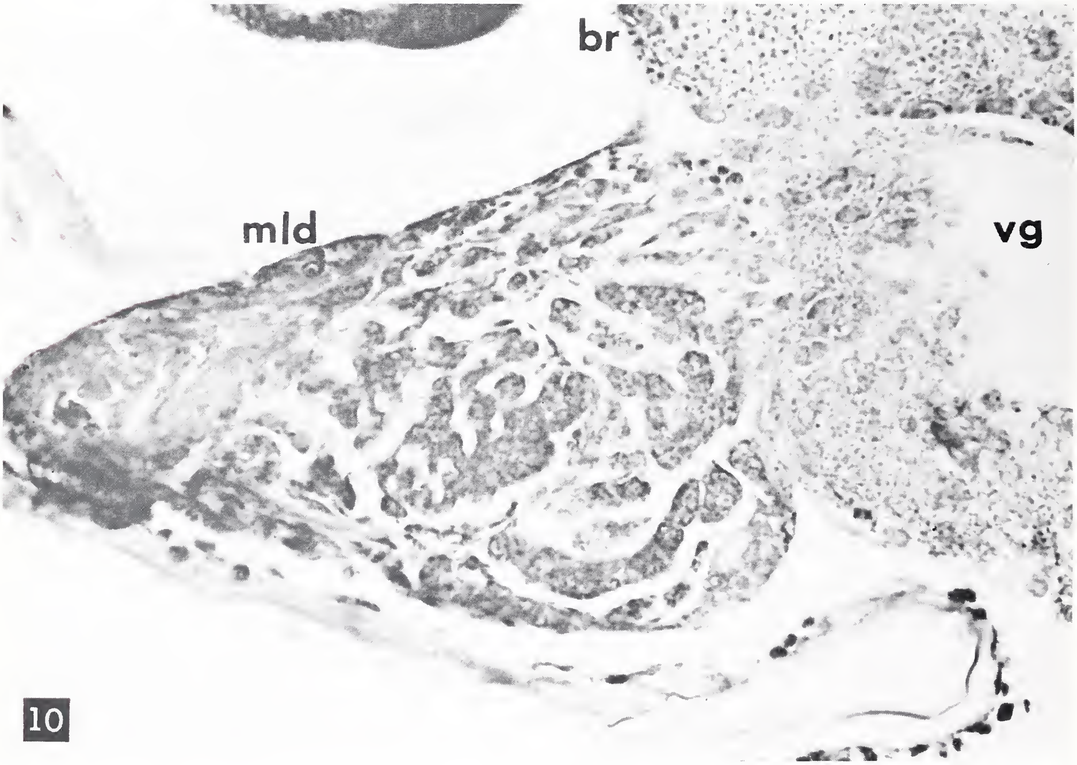


FIGURE 9.—Frontal section through wild-type eye-antennal disc of 96-hour larva. *ed*, eye disc; *ad*, antennal disc; *l*, lumen; and *br*, brain. *Note* folded saclike structure of disc. Hematoxylin and eosin. × 200

FIGURE 10.—Frontal section through first leg disc of 140-hour mutant larva. *mld*, mutant leg disc; *br*, brain; and *vg*, ventral ganglion. Internal organization of *mld* is lost. Hematoxylin and eosin. × 200

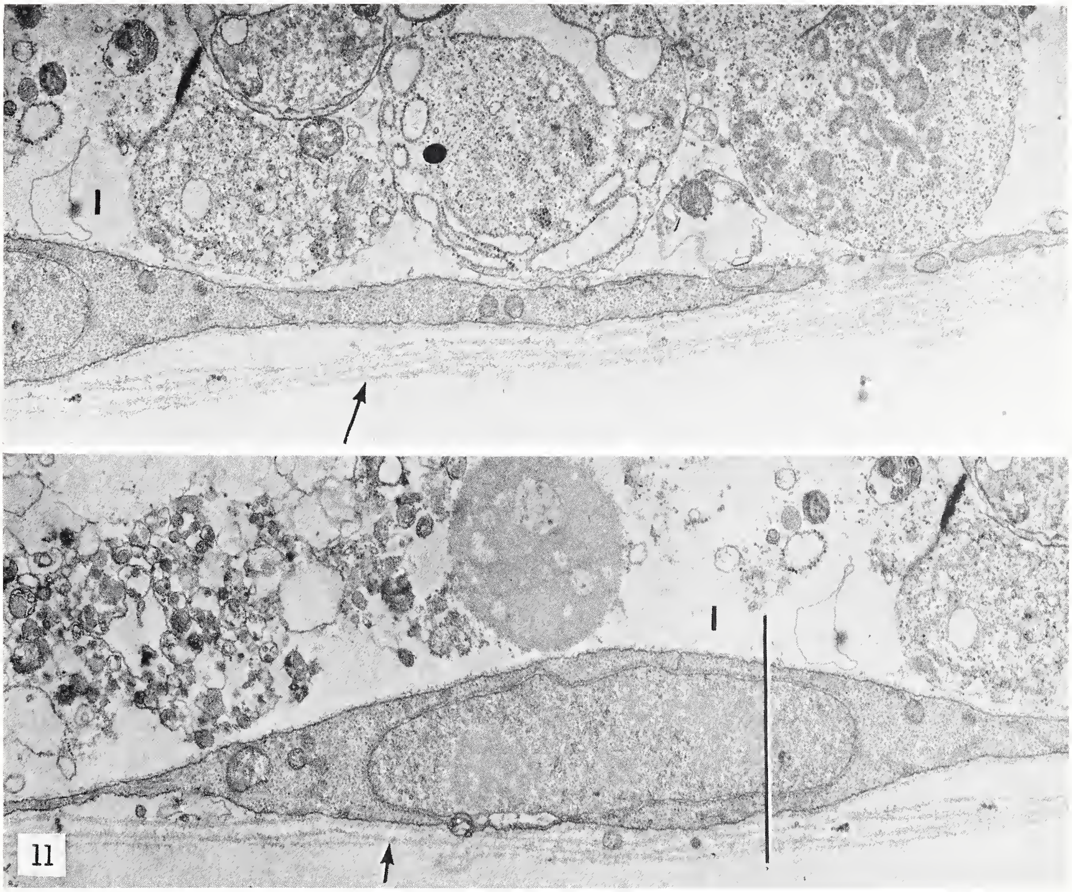


FIGURE 11.—Electron micrograph of peripodial membrane cell of eye-antennal disc of 96-hour wild-type larva. (*Solid line* indicates where the photograph was cut, since the whole cell was too long to be illustrated.) Layers of fine granular material are on outer surface of this flat cell with spindle-shaped cross section (*arrows*). Uranyl acetate and lead citrate. × 18,300

Courtesy of Dr. H. Akai and Dr. L. E. Davis

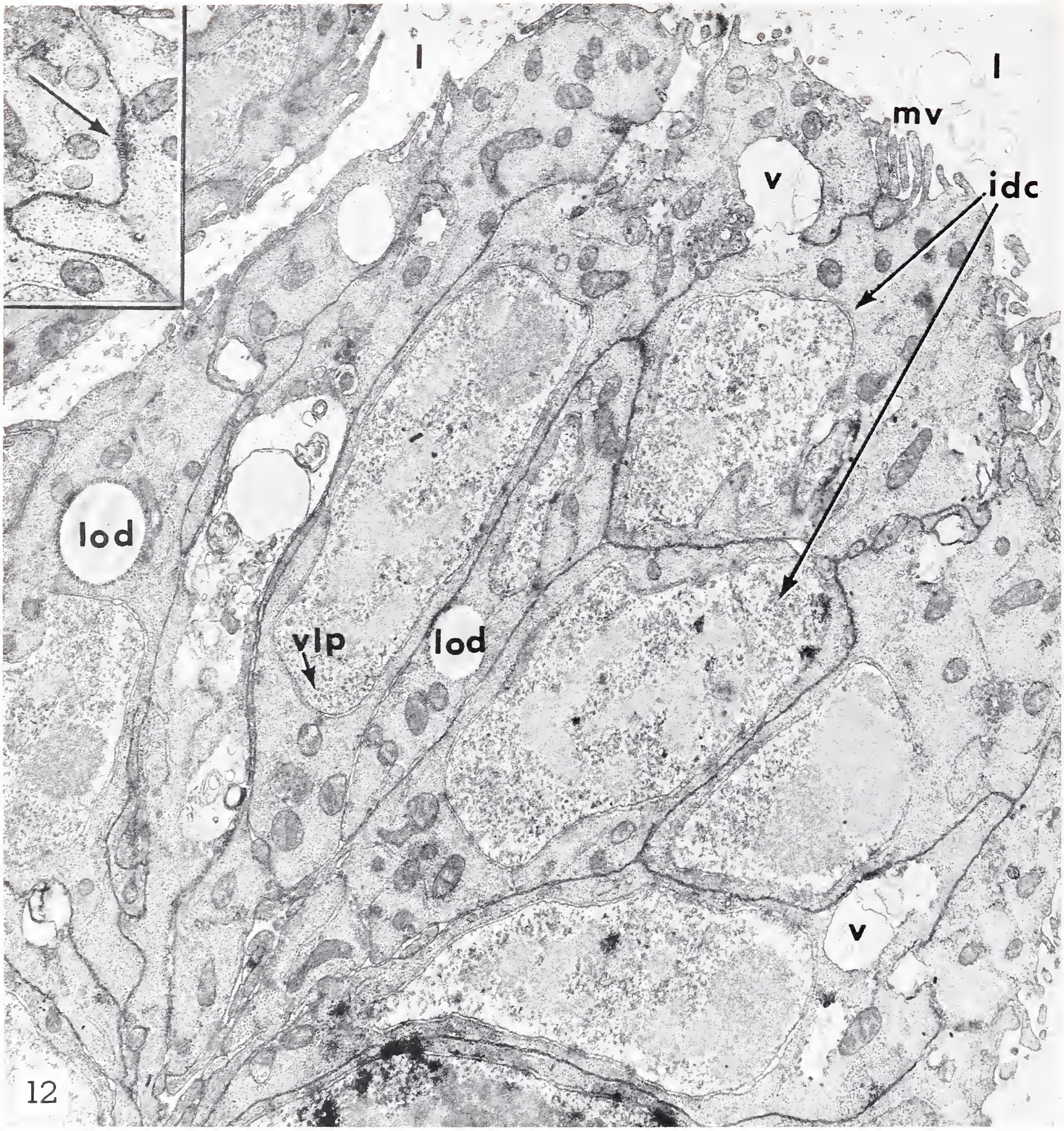


FIGURE 12.—Electron micrograph (detail) of 96-hour eye-antennal disc. (For approximate region *see circle* in fig. 9.) *idc*, imaginal disc cell (*arrows*); *l*, lumen; *lod*, low-opacity droplets; *mv*, microvilli; *v*, vacuole; and *vlp*, virus-like particles (*arrow*). Imaginal disc cells are typically embryonic with *mv* on their luminal surfaces and connect with each other by septate desmosomes (*see arrow* in inset). Uranyl acetate and lead citrate. × 12,500. Inset, × 23,000

Courtesy of Dr. H. Akai and Dr. L. E. Davis

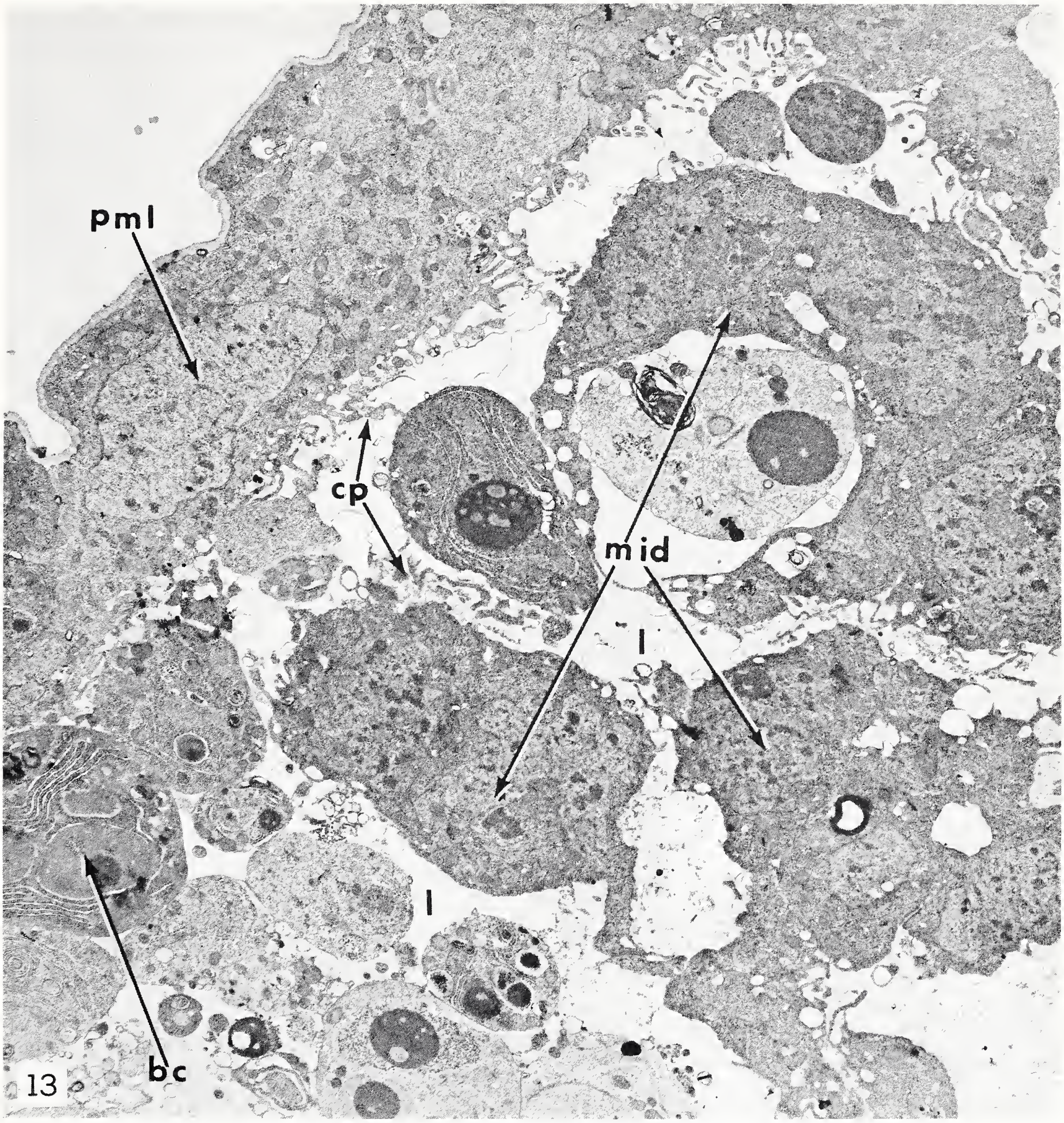


FIGURE 13.—Electron micrograph (detail) of peripheral region of 140-hour mutant eye-antennal imaginal disc. *cp*, cytoplasmic processes; *mid*, mutant imaginal disc (*arrows*); *pml*, peripodial membrane-like cell (*arrow*); *l*, lumen; and *bc*, basophilic cell from lymph gland (*arrow*). Drastically changed in shape and appearance are *mid* cells. Cells have increased electron opacity, and on most of their free surfaces they contain *cp*. In particular, *pml*'s have many of these *cp*. Nuclei vary greatly in shape. Throughout disc are many *bc*'s from lymph gland. Uranyl acetate and lead citrate. × 7320

Courtesy of Dr. H. Akai and Dr. L. E. Davis

Nerve Severance and Tumor Induction in *Periplaneta americana* (L.)[1, 2]

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SUMMARY

Adult female cockroaches were subjected to severance of the esophageal nerve and sacrificed or examined at death at various times after operation. Incidence of tumors was highest in the crop, rising to over 50% by the 5th month. Highly associated with such tumors was a high level of food contents in the crop and also possibly swallowed air. Histologic studies indicated that these crop tumors originated at points of epithelial rupture and muscle insertion. In the latter case, the intima increased in thickness, and the tonofibrils became basophilic. Hemocytes invaded the area and, along with some epithelium, degenerated and added to the basophilic mass. Epithelial cells at the periphery generally increased in size, proliferated, and formed a new epithelium that coursed under the affected area. The basophilic mass was generally pushed into the lumen and the epithelium rose to a normal position. Similar processes occurred in salivary gland tumors and around cellophane implants. It is concluded that these growths were not tumors but the insect defense mechanism for wound healing. Nerve severance and consequent crop wounding and healing generally did not greatly affect the feeding rhythm of cockroaches, but periods of cessation of daily feeding were observed in 60% of the individuals. Possibly the cockroach swallows air to push food through the proventriculus paralyzed by nerve severance.—Nat Cancer Inst Monogr 31: 399–418, 1969.

PREVIOUS LITERATURE on insect tumors induced by nerve severance was recently reviewed (*1*) and also discussed in previous papers in this Symposium. In studying the development of salivary gland tumors in *Periplaneta americana* (L.) induced by duct ligation and duct severance (*2*,*3*), we became interested in severance of the recurrent nerve as a possible method for a comparative study of tumors in such glands. However, in initial studies (*2*) no tumor formation occurred in the glands or alimentary

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[2] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers—The State University.

[3] I gratefully acknowledge the valuable assistance of Mrs. J. M. Chillseyzn.

canal within 1 month after nerve severance, an observation shared by Bodenstein in 1959 (*4*). In subsequent studies, we found that longer intervals generally were necessary for tumor development induced by nerve severance in this species (*5*), a fact also observed by Hema (*6*). The objectives of our continued studies in this area have been to determine the incidence of such tumors, their effect on mortality, their development, and possible cause as related to feeding. The term "tumor" is used with the same reservations expressed by Harshbarger and Taylor (*1*). In the studies, tumor development was allowed to proceed as long as possible to insure a better understanding of such processes.

MATERIALS AND METHODS

Insects.—Adult female *P. americana* from a laboratory colony in culture for over 20 years were used in all experiments. Age at time of treatment was within 2 weeks after molting to the adult. Individuals were immobilized by hypothermia and restrained with a wrapping of thread on a vertical dissecting board. The head capsule was bent forward, and an incision made in the clear dorsal cervical integument. The recurrent nerve, more appropriately termed the esophageal nerve (*7, 8*), lying dorsally on the esophagus, or laterally if the esophagus was distended with food, was readily recognized by the small trachea accompanying the nerve. A short section of the nerve was removed rather than simply severing or rupturing it. Recovery from the operation was improved by sterilizing instruments in ethanol and especially by keeping the cockroaches righted during the first few hours after the operation. Controls generally received sham operations. Cockroaches were maintained in groups of 10, under a light: dark regimen of 12 hours: 12 hours, and supplied with Purina laboratory food. They were either killed and examined at specific intervals thereafter or examined at death, and the nerve severance was confirmed on dissection. To insure that the natural orientation of crop tissues was unchanged for histologic studies, this organ was fixed before removal of food and examination of tumors on its inner surface.

Histology.—Tissues were generally fixed in Carnoy's and stained with Harris' or Ehrlich's hematoxylin and eosin.

Recording of feeding.—To determine the possible influence of nerve severance and tumor development on feeding, cockroaches were confined singly in containers in which were constructed pendulum-type trophometers (*9*). Pushing and pulling on the food affixed to the pendulum opened and closed a circuit, which was recorded on a 20-channel Esterline Angus Recorder. Light: dark conditions were as above, and a cardboard retreat (5.3 × 7.5 × 2.6 cm) was provided.

RESULTS

Description of Tumors

Tumors of the digestive tract exclusive of the rectum are whitish, rather compact, masses as compared to the grayish normal tissue. Compact brown deposits, often occurring within such masses, are readily observed. Based on their location, crop tumors are of three types: 1) 1–3 masses randomly oriented in the middle half of the crop, 2) multiple masses occurring at regular intervals in the crop tissue (fig. 1), and 3) masses occurring at the posterior portion of the crop and its junction with the proventriculus (figs. 2–4). Tumors of the first type often protrude slightly from the surface of the crop and may involve a small amount of fat body. Tumors of the latter two types generally do not protrude from the crop surface. Against a background of food within the crop, these tumors, if small, can be overlooked. It is necessary, therefore, to remove the food before examination for tumors. When the inner crop surface is examined, generally spherical or star-form deposits of brown material occur at or near the center of the surface of tumors. In a few treated individuals, tracheae on the surface of various parts of the digestive tract had undergone "melanization." Although sometimes associated with a tumor, this process by itself in a trachea was not considered tumorous in these studies. Tumors of other portions of the digestive tract generally did not protrude from the organ surface, except for a few proventricular tumors.

Sites and Incidence of Tumors

The sites and incidence of tumors in the alimentary tract of 217 cockroaches subjected to nerve severance are given in table 1. The data are based on sacrificed and dead individuals whose tissues were still suitable for examination. The crop was the major site of tumor formation, the incidence in this organ rising to approximately 50% in 5 months. Those tumors designated in the "remaining digestive tract" (exclusive of the rectum) include an average of 3% in the proventriculus, 3% in the coeca, 6% in the midgut, and 11% in the hindgut. In 119 control individuals of age similar to that of individuals in treated groups, incidence of tumors was in the crop 0%, in the salivary glands 5% (range 5–15%), in the proventriculus 1%, in the coeca 1%, and in the hindgut 1%.

Crop Tumor Association With Abdomen Distension

Initially in these studies it was observed that generally 1 month after operation the abdomens of some individuals were distended or swollen to the point that the white fat body was easily observed in the intersegmental membranes and pleura. In extreme cases hemolymph exuded from such areas. Often movement was impeded and such individuals were killed for examination. Invariably this condition was due to an abnormally large quantity of food in the crop, representing as high as 25% of the weight of the cockroach. In a few cases, distension, which was eliminated by brief hypothermia before dissection, was apparently due to a large quantity of swallowed air in the crop. To determine the possible relationship of crop tumors and quantity of food in this organ, on dissection the level of food in the crop was judged arbitrarily as "full" or "not full." The association between the percent incidence of crop tumors and percent of individuals having "full" or "not full" crops is also given in table 1. The average incidence of crop tumors in individuals with "full" crops was 62%, that for individuals with "crops not full," 21%.

TABLE 1.—Sites and percent incidence of tumors in adult female *Periplaneta americana* at various times after nerve severance, and association of tumors with level of crop contents

Groups	Months after nerve severance							
	0.5–1	2	3	4	5	6	7–8	9+
Percent tumorous:								
In crop	25	30	37	42.9	54.6	36	50	40
In salivary gland	0	10	2.9	2.4	9.1	16	7.9	4
In remaining digestive tract	20	10	17	19.1	27.3	12	31.6	32
Percent with full crop	30	20	45.8	66.7	54.6	40	65.8	28
and crop tumorous	20	10	25.7	53.6	40.9	24	39.5	28
Percent with crop less than full	70	80	54.2	33.3	45.4	60	34.2	72
and crop tumorous	5	20	11.4	7.1	13.7	12	10.5	12
Numbers of killed and dead individuals examined	20	10	35	42	22	25	38	25

Histology and Development of Crop Tumors

Extensive studies of the histology of crop tumors in various stages of development indicated that such tumors develop in the following manner. Initially, at the site of tumor formation the basal layer of the crop intima becomes basophilic and the intima increases some 4–5 times in thickness. These processes occur at and around the point of insertion of longitudinal and circular muscles (figs. 5 and 6), and the basophilia spreads through the thickened cuticle and tonofibrils of the muscle insertion. Generally the nuclei of epithelial cells in the immediate area become pyknotic and the cells become misshapen. The cells degenerate and add to the basophilic mass (figs. 7–9). During this time, hemocytes invade the area beneath the affected epithelium and encapsulate the proximal portion of muscles involved in the insertion (figs. 7–9). Generally, if the insertions of the circular muscles are not involved, such muscles are encapsulated little, if at all, and these muscles cover the encapsulated area.

The hemocytes assume an orientation around the affected area, but not in the definite swirl pattern observed in salivary gland tumors (*3*). Those hemocytes closest to the basophilic mass progressively degenerate and add to the mass (figs. 7–9); no mitotic figures have been observed among such cells. At this time or slightly later, those unaffected epithelial cells (figs. 7 and 8) at the periphery of the affected area increase in size and undergo mitosis, forming a new epithelium which courses through the distal layers of encapsulating hemocytes (fig. 10). This new epithelium invariably is associated with a somewhat discontinuous, thin, basophilic layer just proximal to the new epithelium. It is not known if this is a product of such epithelium or if the encapsulating hemocytes form this to orient the new epithelium as it becomes continuous with surrounding epithelium.

In the interim, those hemocytes central to the area of new epithelium continue to degenerate and add to the basophilic mass. Toward the center of the mass, compact, brown material forms (figs. 9 and 10), similar to inclusion cyst material in gland tumors (*3*). It is apparently derived from the basophilic mass. This material increases in size and becomes hard and brittle, and eventually it and the surrounding basophilic mass or scab are pushed toward and sloughed into the crop lumen (figs. 11 and 12). Concurrently, the hemocytes peripheral to the new epithelium reenter the hemolymph circulation (fig. 12). With the sloughing of the scab, the new epithelium rises to the level of the surrounding epithelium and possesses a slightly thickened intima, which initially at least is faintly basophilic with characteristic intima spicules (fig. 11). In some cases portions of this intima are tanned. The original muscle insertion is destroyed though the muscle itself is not. Such tumors may be so numerous (fig. 16) that much of the muscularis is freed from the crop wall (fig. 4).

A modification of this process has been observed when a tear in the epithelium (fig. 13) occurs near the muscle insertion. Hemocytes accumulate at such an area and apparently block the escape of crop contents into the hemolymph. The hemocytes eventually are destroyed, yielding a granular mass protruding into the hemolymph (crop tumor, type 1). A small portion of fat body may be attached and encapsulated, and contribute to the mass.

Small, intensely basophilic particles of irregular shape and size are evident in certain portions of the mass (fig. 14); the source of these particles is the nuclei of lysed hemocytes. New epithelium generally does not course around such masses. In fact, the peripheral epithelium may be destroyed (fig. 14). New epithelium may be lacking in some tumors (fig. 15). In a few individuals portions of the crop wall were void of epithelium.

Tumors of the proventriculus, which appear to be continuous with those at the basal portion of crop, develop in a manner similar to those in the crop. The layer of encapsulating hemocytes is thicker, but subsequently the basophilic mass is pushed into the lumen as a new epithelium forms. Incidence of midgut tumors has been too low to study their development. Hemocytes are involved, and generally there is an abnormal rate of exfoliation of midgut cells into the lumen. Tumors of the hindgut likewise involve hemocytes and also production of inclusion cyst material. In one hindgut tumor this material was pushed into the lumen as a new epithelium was formed. Tumors of the salivary gland were similar to those induced by duct ligation, including processes of hemocyte encapsulation and formation of vesicles derived from intercalated duct cells (*3*).

Effect of Nerve Severance on Mortality

Accumulative percent mortalities of 212 cockroaches subjected to nerve severance and 101 control individuals from 1 month after treatment are given in table 2. Mortalities during the 1st month are not included so as to eliminate the influence of mortality that sometimes occurred within the first 2 weeks after treatment. The figures are somewhat biased, since in some groups, from which these figures were derived, often 10–20% of the individuals were sacrificed to determine tumor incidence. These were generally those individuals with distended abdomens that were judged incapable of surviving. Figures for the controls are likewise biased, since individuals in some control groups were sacrificed after the 10th month. The data do indicate, however, that, among treated and untreated individuals dying after the 1st month, the rates of mortality are very similar. Only after the 4th month, a time of maximum incidence of crop tumors (table 1), does mortality among treated individuals increase slightly above controls. This fact, in addition to the leveling off of crop tumor incidence in the 5–9 months after nerve severance (table 1), supports the conclusion that nerve severance and tumors induced by it do not greatly increase mortality.

Effect of Nerve Severance and Tumors on Feeding

A summary of the effect of nerve severance and tumors on the feeding of 15 individuals is given in table 3. The feeding graphs (trophographs) for individuals 1–5 are shown in text-figures 1 and 2. On the left of each trophograph are indicated the months after feeding records were initiated. On the right margin of each graph is indicated the condition of abdomen distension (O, normal; H, partially distended; S, very distended and swollen) at that time. At the top of each graph, a solid black bar indicates the 12-hour period of darkness in the 24-hour day. The time of nerve severance is indicated by a black arrow. All individuals were supplied a retreat except for number 1, whose retreat was removed from the 2d to the 4th week, under which conditions some daytime feeding was observed.

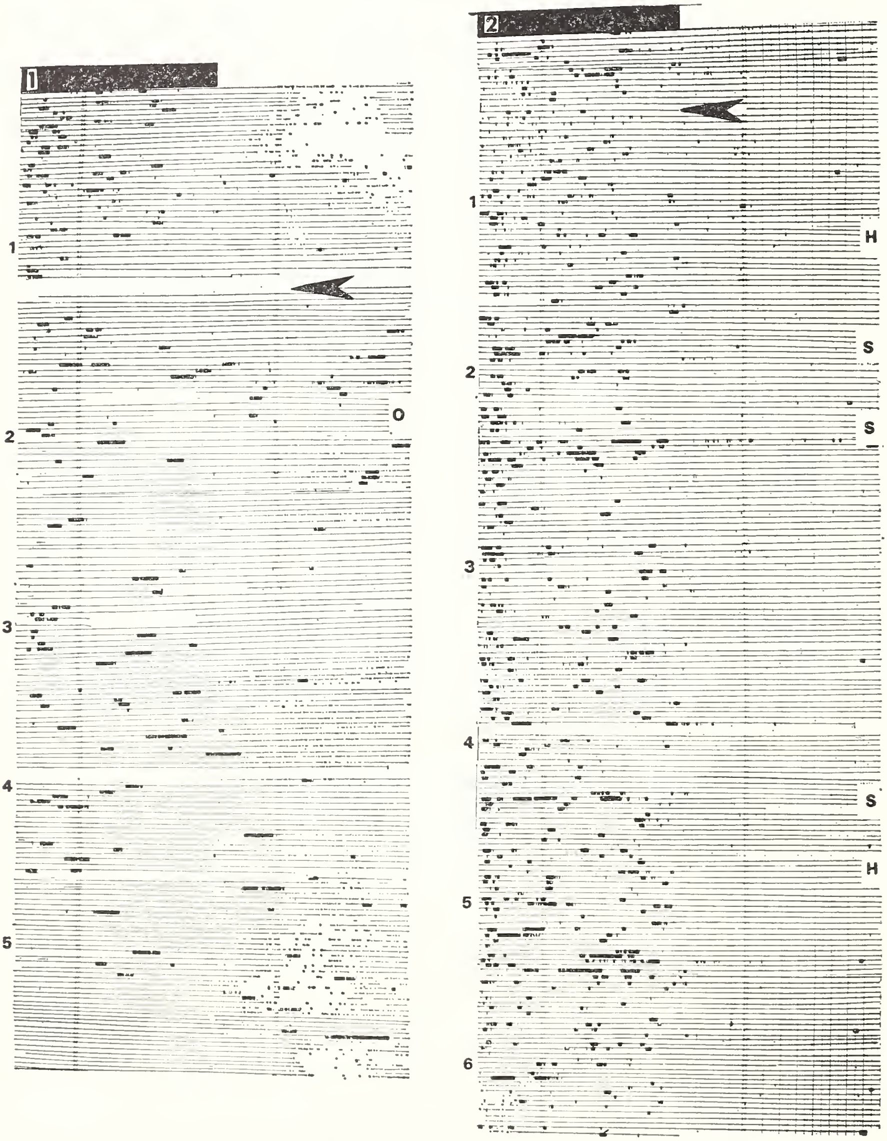
TABLE 2.—Accumulative percent mortalities of treated and control cockroaches at various months after nerve severance

Treatment	Months															
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Nerve severance, 212 individuals......	13	27	50	63	75	84	87	93	96	97	98	98	99	99	99	100
Control, 101 individuals.	20	33	53	60	68	80	84	90	93	95	98	98	99	100		

TABLE 3.—Summary of effects of nerve severance and tumor formation on cockroach feeding

Cockroach	Feeding interval monitored after nerve severance (months)	Fate	Level of food content in crop	Site of tumors	Effect of feeding
1	4.75	Died	Normal	Crop	Rhythm disrupted; feeding often ceased for up to 5 days, duration of feeding periods increased.
2	6	Killed	Very full	Crop	Rhythm not affected; daily feeding; duration of feeding periods normal.
3	5.5	"	Very full	Crop	Rhythm not affected, secondary nocturnal feeding decreased during 3d month after operation, feeding in light decreased during 5th month after operation; daily feeding; duration of some feeding periods increased.
4	3.4	"	Very full with air	Crop hindgut	Rhythm not affected; daily feeding; duration of feeding periods increased.
5	2.5	Died	Normal	Not examined	Rhythm shifted to light period until death.
6	5.3	Killed	Very full	Crop	Rhythm not affected; daily feeding; duration of feeding periods normal.
7	6	"	Normal	None	Rhythm not affected, interval between nocturnal feedings increased; daily feeding; duration of feeding period normal.
8	5.3	"	Normal	Crop hindgut	Rhythm not affected; daily feeding except 15 days in 4th month after operation; duration of feeding periods normal.
9	2.7	"	Very full	Crop proventriculus	Rhythm disrupted; feeding often ceased for 2 days; duration of feeding periods normal.
10	5.3	"	Normal	Crop	Rhythm not affected; feeding often ceased for 2 days; duration of feeding periods normal.
11	2.5	"	Very full	Crop	Rhythm not affected; periodically ceased feeding; duration of feeding periods normal.
12	2.5	"	Very full	Crop	Rhythm disrupted; ceased feeding for 5 days in 2d month; duration of feeding periods normal.
13	2.5	"	Very full	Crop	Rhythm only slightly disrupted, secondary nocturnal feeding occurred later; ceased feeding for 5 days in 2d month; duration of feeding periods normal.
14	2.5	"	Normal	None	Rhythm not affected; daily feeding; duration of feeding periods normal.
15	2.5	"	Very full	None	Feeding reduced for 2 months after operation, resumed last half month; duration of feeding periods normal.

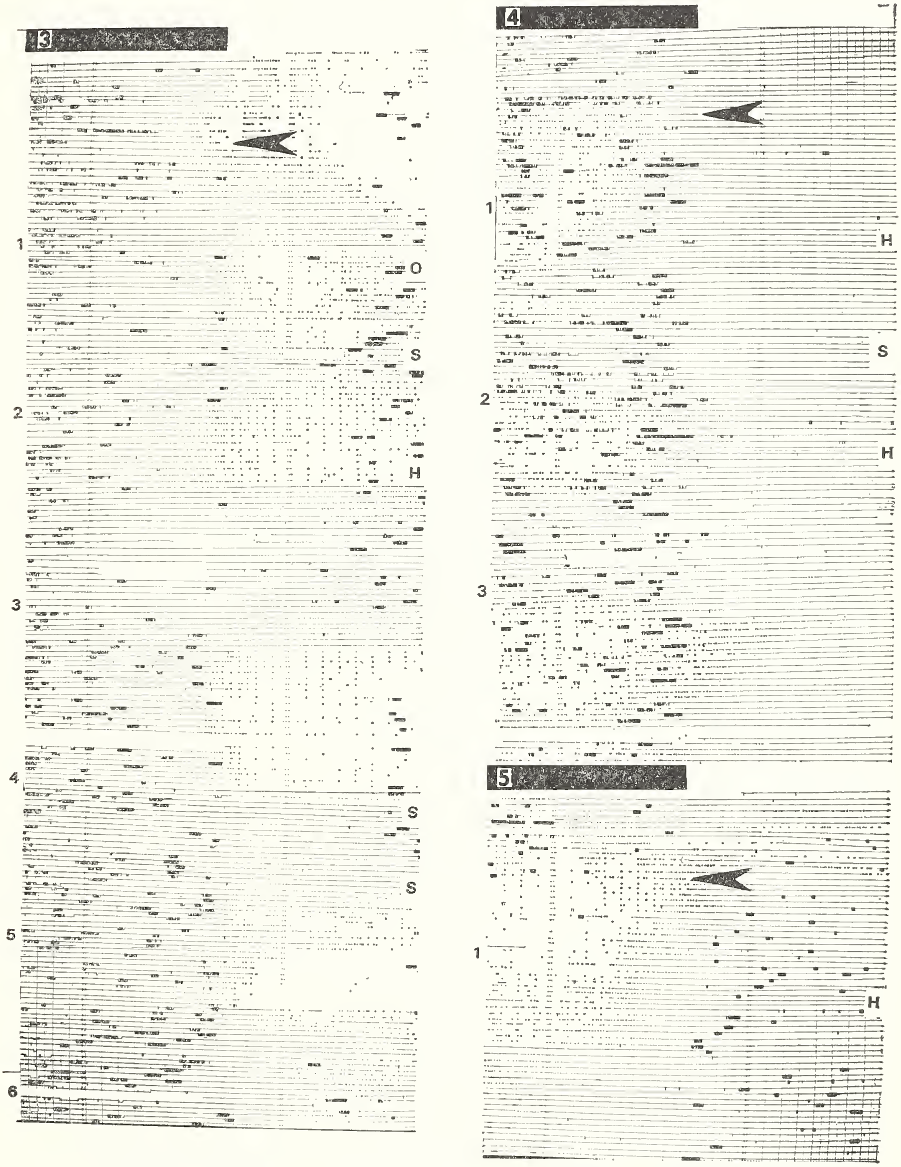
In concurrent studies with control cockroaches, it has been observed that cockroaches are generally nocturnal feeders, feeding 1–3 times each night under entrained conditions of 12:12 light:dark. Of the 15 individuals subjected to nerve severance, the rhythm of nocturnal feeding was maintained in 60%. In the other 40%, rhythms were disrupted to varying degrees. Periods of cessation of daily feeding were observed in 60% of the individuals. Although the trophometer does not record quantitatively the amount of food consumed, most individuals remained at the food for a normal length of time. In the few cases where duration of feeding was increased, trophograph tracings were not as compact, possibly



TEXT-FIGURE 1.—Trophographs of two cockroaches subjected to nerve severance.

indicating that the individual desired to feed but had difficulty in ingesting more food into a crop apparently already distended with food (*e.g.*, # 4).

In 60% of the individuals, the crops were very full with food, or swallowed air, or both, at the time of dissection. In most, abnormal distension and swelling of the abdomen were not permanent symptoms. A reduction in these symptoms, however, was not associated with a marked cessation of feeding. The incidence of crop tumors was 78% among those examined.



TEXT-FIGURE 2.—Trophographs of three cockroaches subjected to nerve severance.

DISCUSSION

The severance of the esophageal nerve in insects undoubtedly interferes with the normal functioning of the tissues it supplies. In the blowfly, *Phormia regina*, such an operation interferes with stretch receptor mediation of the quantity of food consumed, with the result that the flies overfeed and the foregut is ruptured (*10*, *11*). It has been reported that nerve severance prevents passage of food from the crop to the midgut in *Leucophaea maderae* (*12*), possibly due to paralysis of the proventriculus, which occurs after nerve severance in *P. americana* (*13*). In our studies with the latter species complete cessation of food passage into the midgut has not been observed. However, within a few months after nerve severance, the level of crop contents generally increases apparently due to a relatively normal ingestion of food, as indicated by feeding studies, coupled with a less than normal rate of release of food into the midgut. This species normally swallows air during the period of crop emptying, thereby maintaining a gradient of pressure across the proventricular valve (*14*). On our studies, hypothermia before dissection was observed to release such crop air, and, therefore, the incidence of food in the crop (table 1) may not give a true indication of the actual distension of the crop by air as well as food. In several cases, however, abnormal abdomen distension was due only to such air, and it is very probable that after nerve severance the cockroach employs this process in an effort to force food past the paralyzed proventricular valve.

Strongly associated with high levels of crop food content after nerve severance is the development of tumors in the crop. Based on their histology, these growths are in response to the tension on muscle insertions in the crop wall and small ruptures in the intima and epithelium. The hemocytic encapsulation and the general repair of the epithelium in such growths are similar to wound healing in this and other species (*15*, *16*). In *Rhodnius prolixus* (*16*), the steps in wound healing of the integument are very similar to the healing of the crop wall described in this study, except that the muscle insertions in the crop are destroyed.

In the wound-healing processes of *R. prolixus* and the larvae of *Ptychopoda seriata* (*16*, *17*), giant cells were observed, which apparently disintegrated or disappeared. In current studies of wound healing in the crop, no such cells were observed, though the epithelial cells increased in size. In salivary gland tumors induced by duct ligation, giant cells, 30 μ in diameter, appeared within the tumors (*3*). They originated apparently from the cells of the intercalated ducts and ultimately underwent division to form a new vesicular epithelium.

New epithelium is also formed around pieces of cellophane or celloidin implanted in *P. americana*, *L. maderae. Locusta migratoria*, and *Oryctes nasicornis* (*3*, *17–20*). Generally the source of this epithelium is the epithelial cells of tracheae adjacent to the implant. Giant cells have been observed (*3*), their source being probably the tracheal epithelium.

Since the general processes and results following implantation are similar to those in gland and crop tumors in *P. americana*, all such growths should be labeled as processes accompanying wounding and healing. These processes are examples of the efficient defense mechanism of this species to seal off foreign bodies and affected areas, temporarily with hemocytes and permanently with a layer of new epithelium. The proliferation of the latter is controlled growth, and this process and hemocyte encapsulation should not be considered tumorous. Schlumberger in 1952 (*21*) was of a similar opinion concerning tumors induced in *L. maderae* by nerve severance. It would be of interest in future studies to determine if the new epithelium might become hyperplastic in the absence of encapsulating hemocytes which, in the wound reactions, appear to act as a mold. Since hemocyte encapsulation could mask true insect tumors, interfere with their histological interpretation, or act as a defense mechanism against tumor development, future studies should investigate methods to reduce hemocyte levels or their mechanism and efficiency of encapsulation.

At the present time it is not known if crop tumors in other cockroach species are similar to the crop wound reactions in *P. americana*. Possibly, in other species, tumor development or wound repair occurs at a slower rate. Therefore, histological studies of late tumor development are needed for further clarification. However, additional species or strain differences may be involved. For example, in a recent study with *Blattella germanica*, severance of the esophageal nerve did not increase tumor incidence above that in controls (*22*). In contrast to our results with *P. americana*, Hema (*6*) has observed the greatest effect of nerve severance in the midgut and salivary reservoirs. Such differences may depend on the type of food offered the cockroach. Solid dry laboratory food may pass through the paralyzed proventricular valve at a much slower rate than a semifluid food such as banana. In addition, the conditions under which cockroaches are maintained may influence the amount of food ingested. In recent experiments we have observed that, at certain times in the adult life, cockroaches under a 12:12 dark:light regimen will consume more food in the absence of a retreat.

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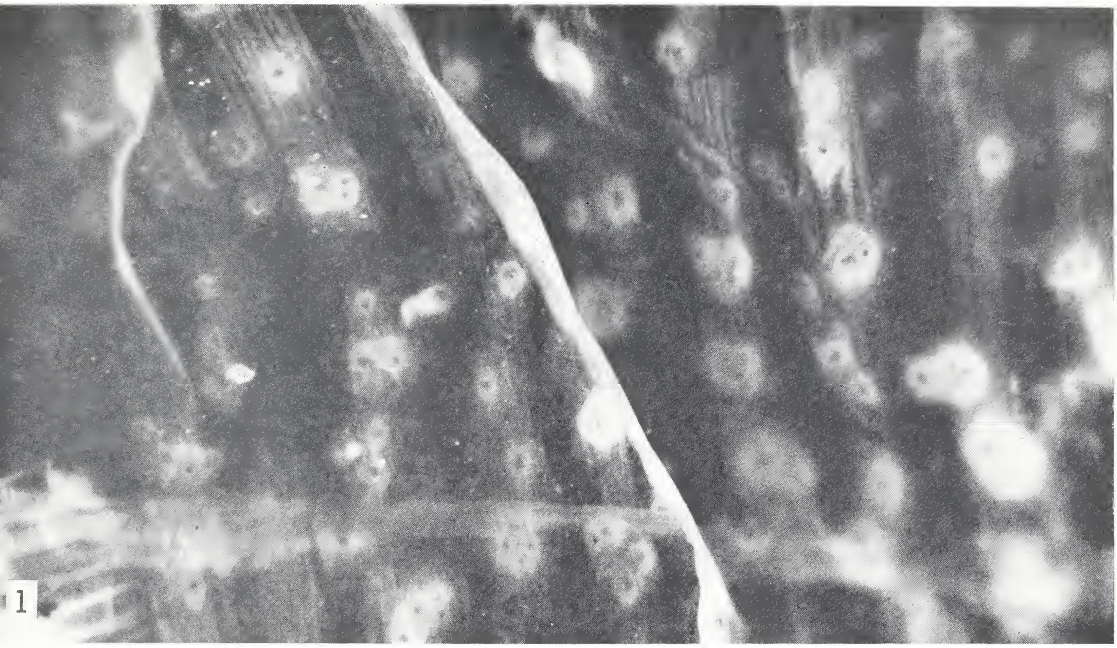


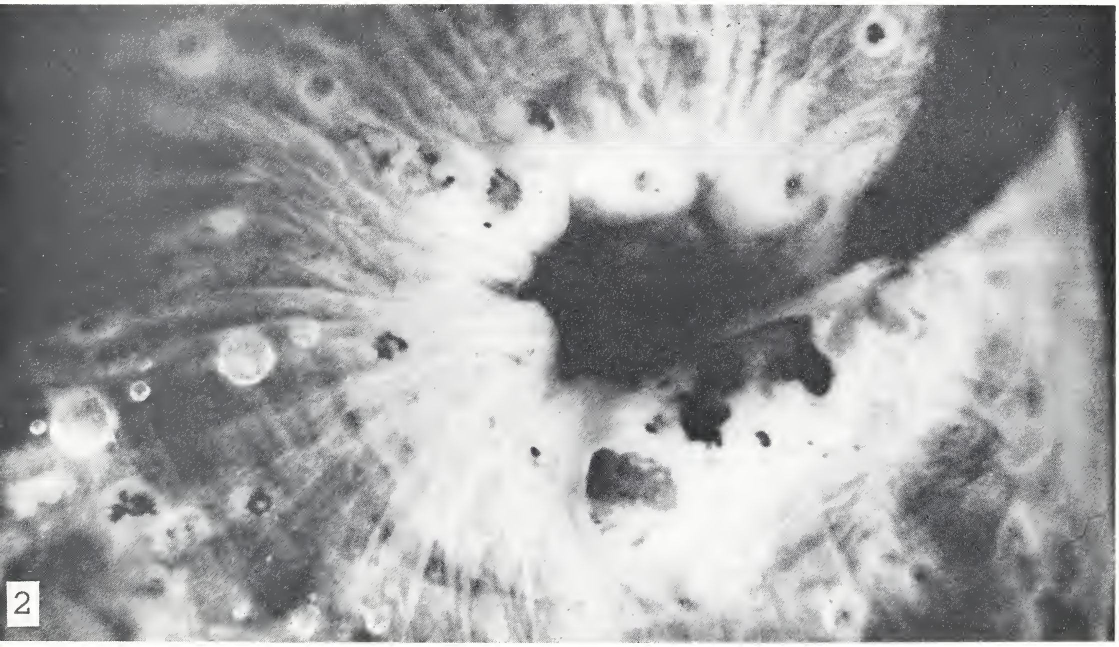
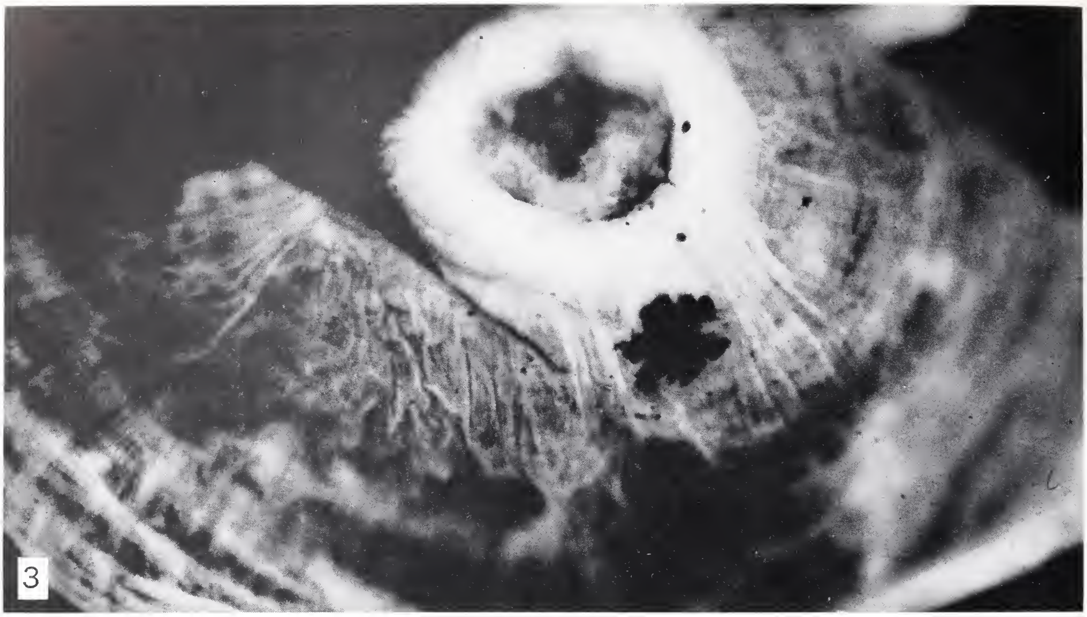


FIGURE 1.—Time after nerve severance, 3 months. Small multiple tumors occurring at insertions of circular muscles. Fixed tissue. × 64

FIGURE 2.—Time after nerve severance, 3 months. Tumors in the basal portion of the crop and its junction with proventriculus. Internal view, fixed tissue. × 64



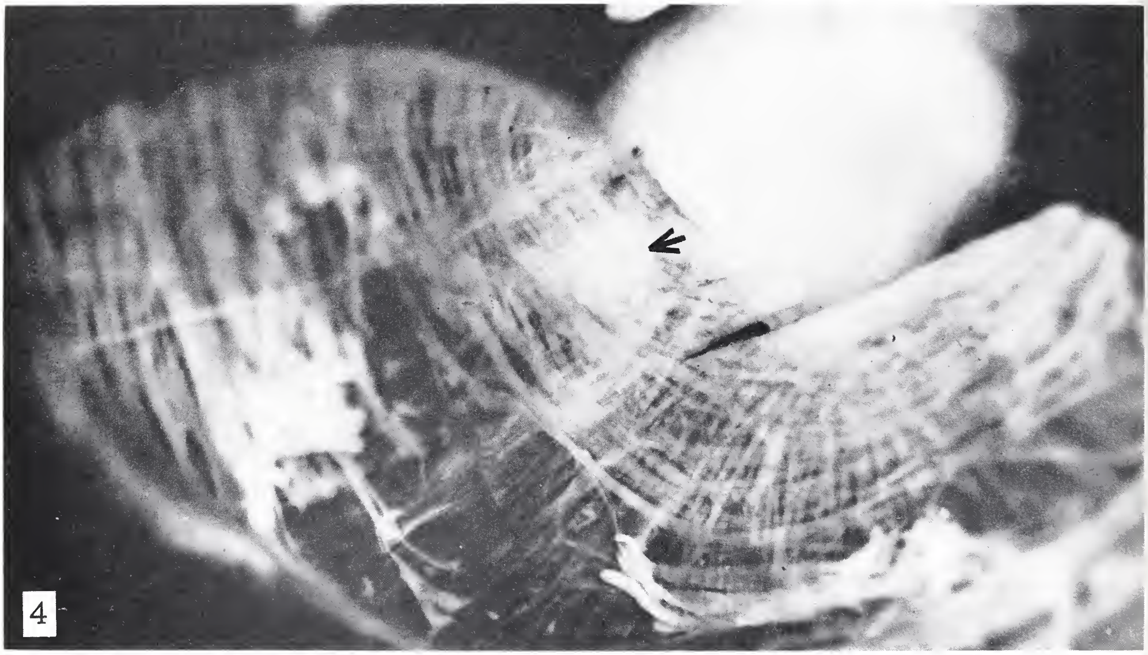
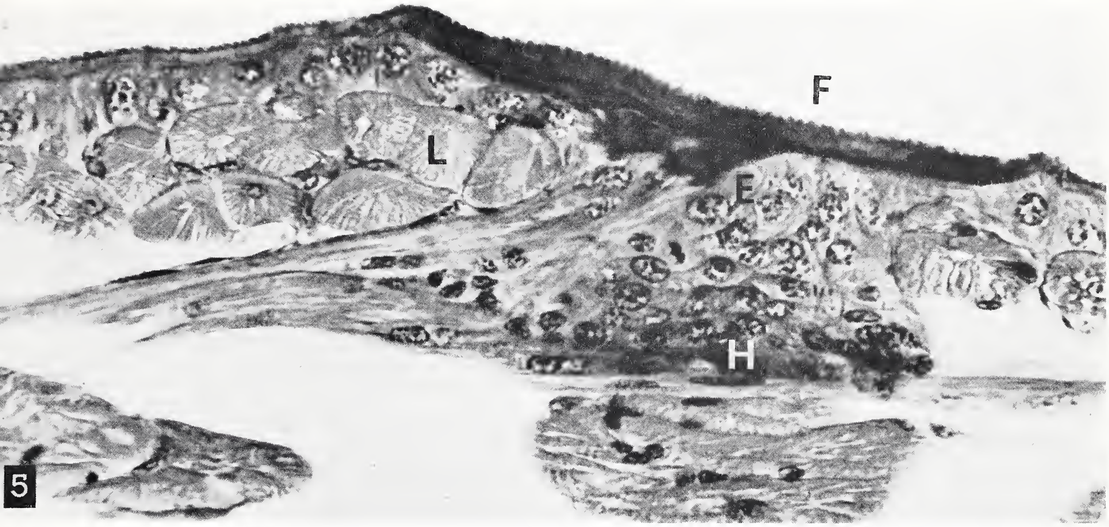


Figure 3.—Time after nerve severance, 5.3 months. Large tumor in the basal portion of crop. Internal view, fixed tissue. × 64

Figure 4.—Same as figure 3. External view with tumor (*arrow*). External circular muscle detached from crop wall. Fixed tissue. × 64



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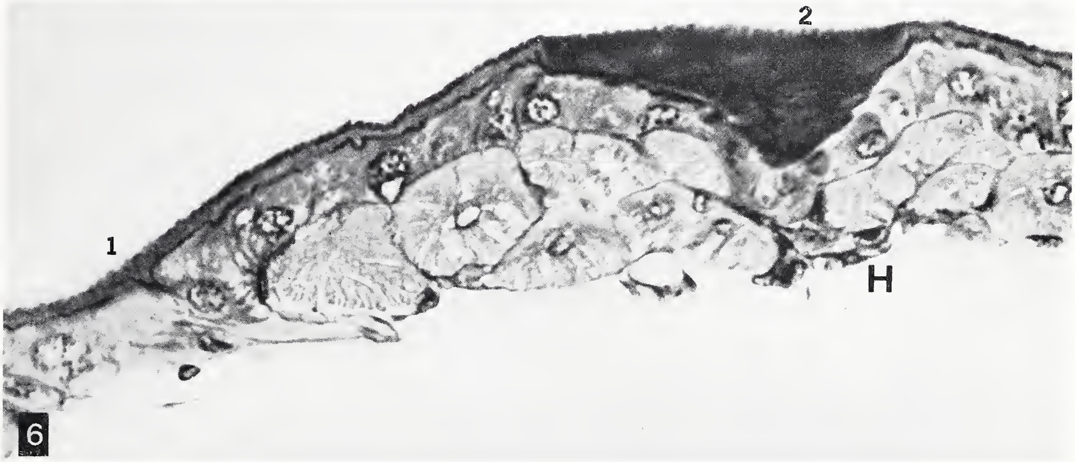

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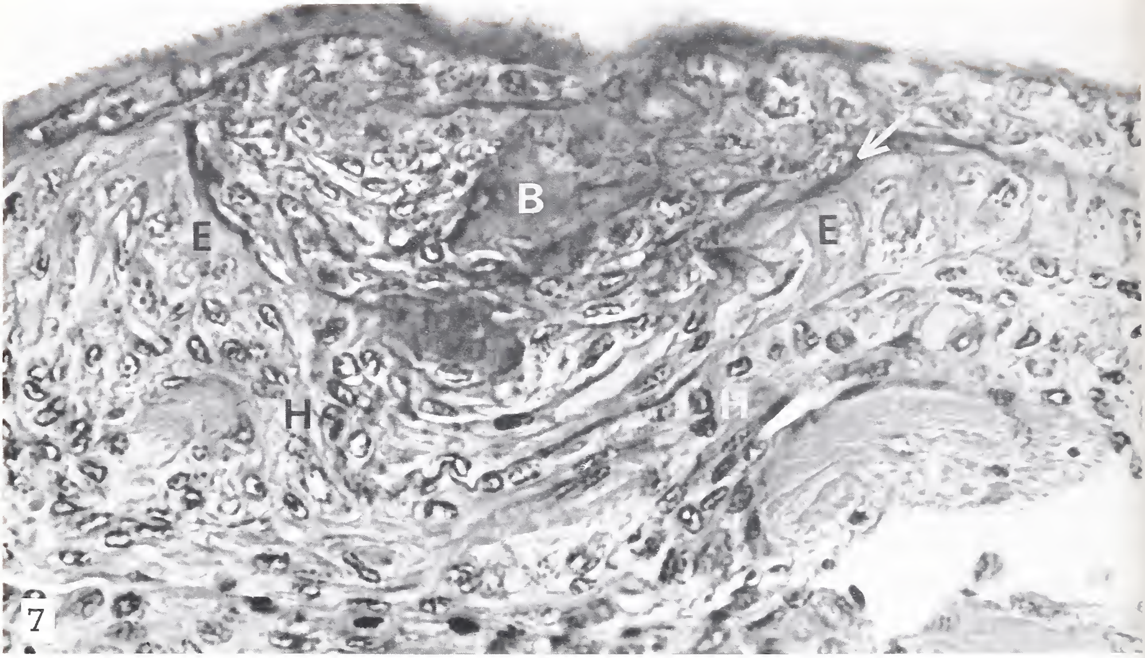
Figure 5.—Time after nerve severance, 2.5 months. Intima and tonofibrils at insertion of circular muscle becoming basophilic. Hemocytes (H) invading the area, and some mitotic activity in the epithelium (E). Inner longitudinal muscles (L) and crop lumen (F). Hematoxylin and eosin. × 560

Figure 6.—Time after nerve severance, 2.5 months. Two sites of muscle insertion (1, 2) with basophilia appearing in 2 and the basal layer of intima. Hemocytes (H). Hematoxylin and eosin. × 630



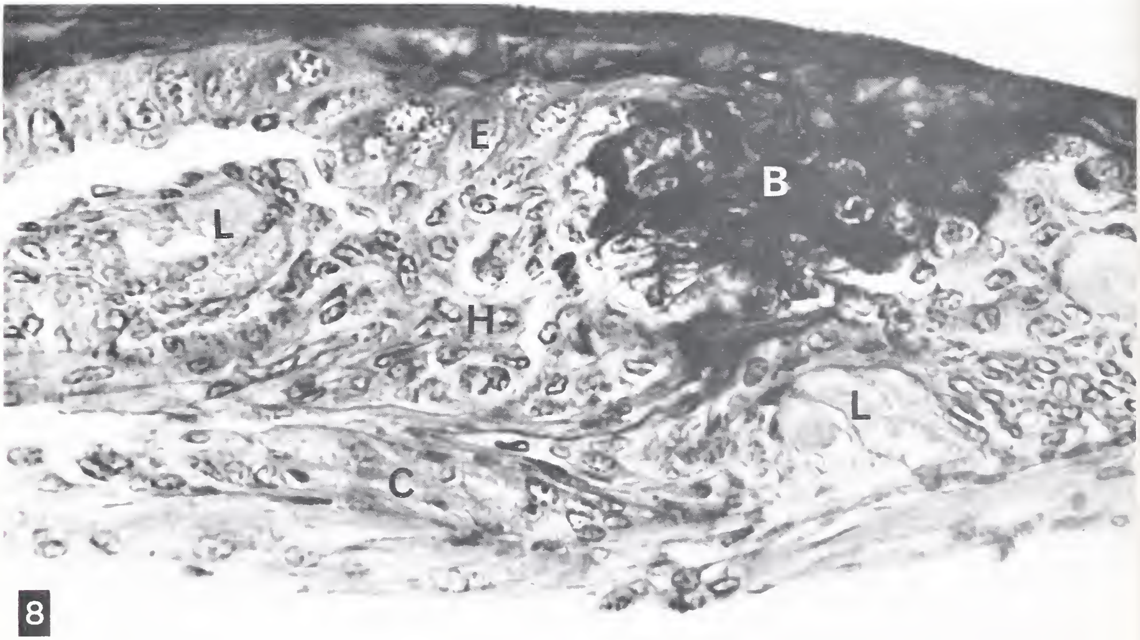
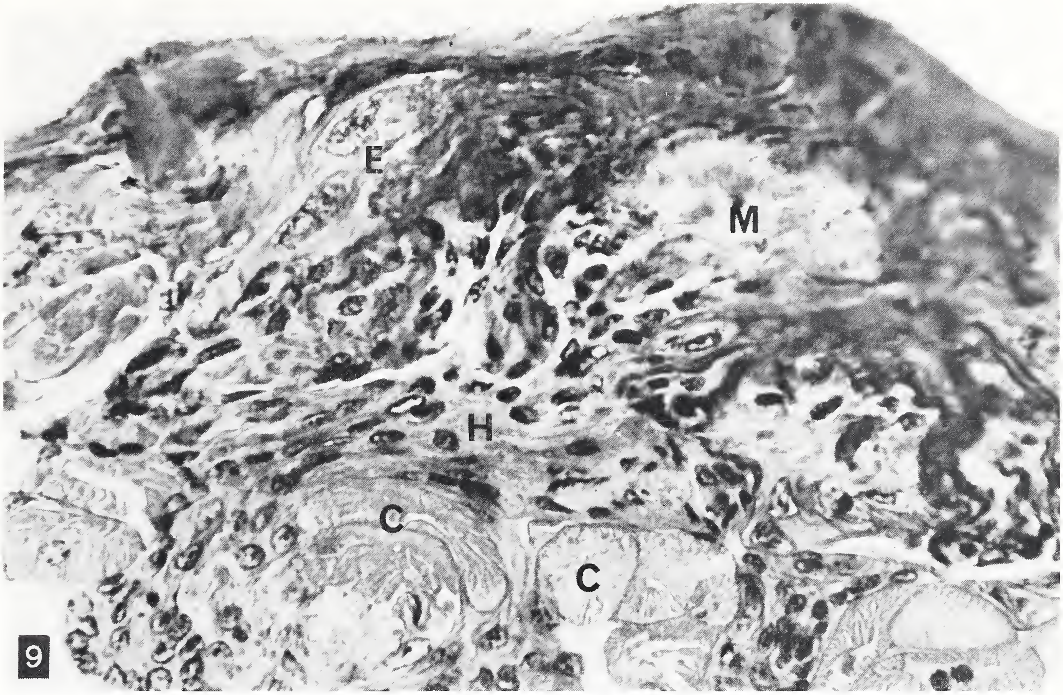


FIGURE 7.—Time after nerve severance, 2.5 months. Hemocytes (H) encapsulating the area, and proliferation of new epithelium (E). Cells above are adding to the basophilic mass (B). Basophilic layer next to epithelium (*arrow*). Hematoxylin and eosin. × 630

FIGURE 8.—Time after nerve severance, 2.5 months. Hemocytes (H) encapsulating the area and adding to the basophilic mass (B). Epithelial cells at margin increasing in size (E). Circular muscles (C), longitudinal muscles (L). Hematoxylin and eosin. × 630



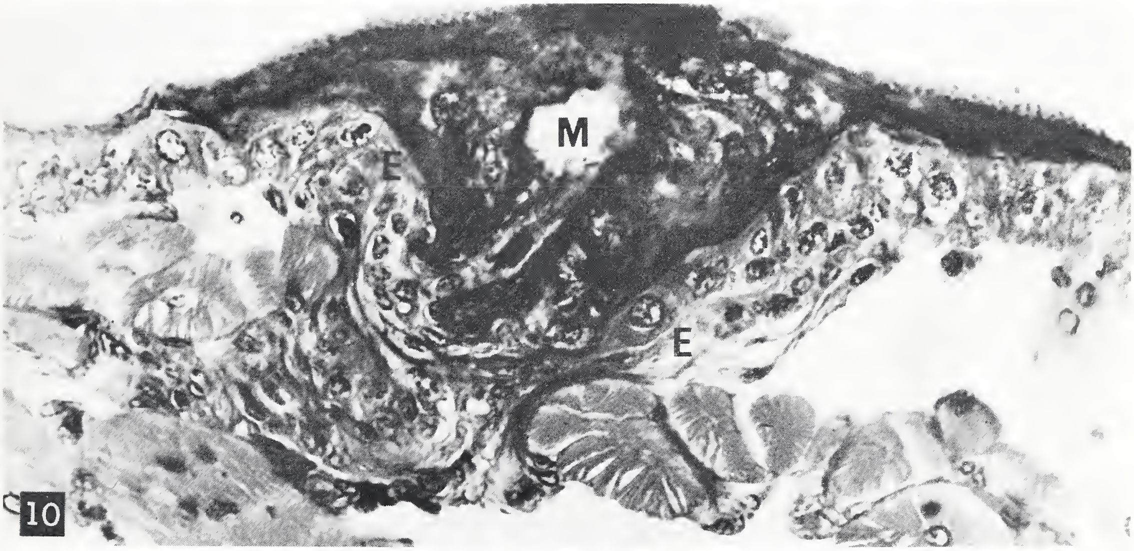
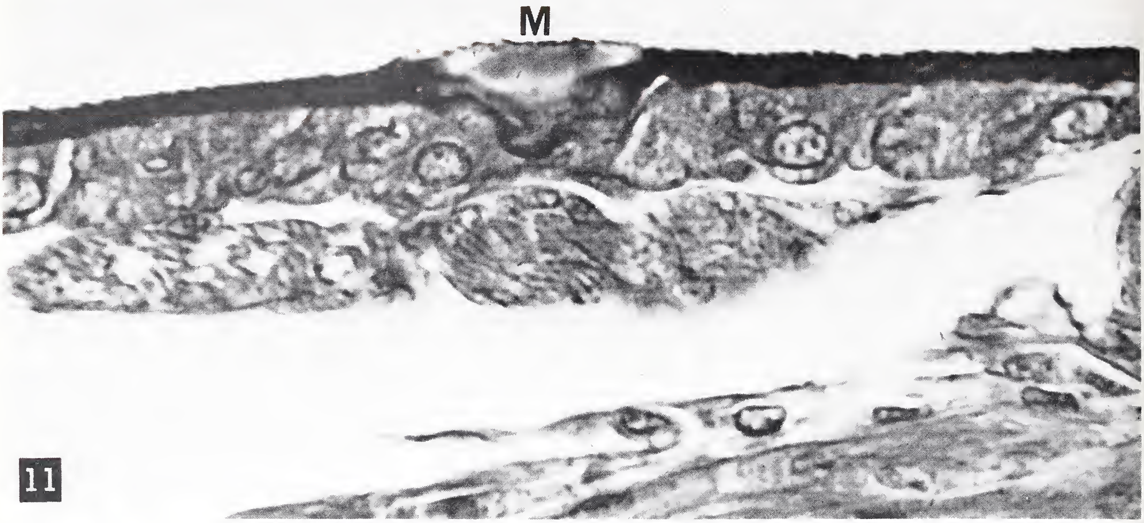


FIGURE 9.—Time after nerve severance, 7 months. Hemocytes (H) encapsulating circular muscles (C) and contributing to the basophilic mass, in the center of which is forming inclusion cyst material (M). Two remaining epithelial cells (E) are increasing in size. Hematoxylin and eosin. × 630

FIGURE 10.—Time after nerve severance, 2.5 months. New epithelium (E) coursing beneath affected area. Inclusion cyst material (M). Hematoxylin and eosin. × 560



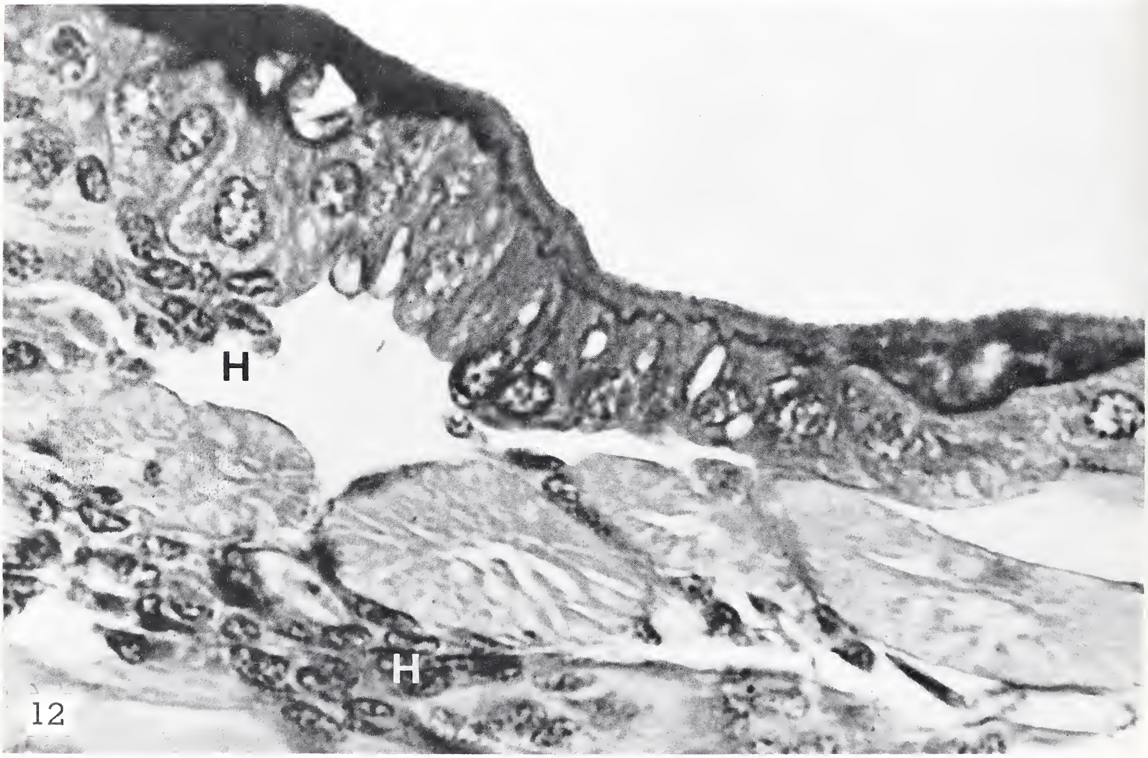


Figure 11.—Time after nerve severance, 2.5 months. Scab of inclusion cyst material (M) pushed toward lumen and epithelium almost at normal level. Hematoxylin and eosin. × 980

Figure 12.—Time after nerve severance, 3 months. Similar to figure 11 with remaining hemocytes (H) reentering circulation. Hematoxylin and eosin. × 980

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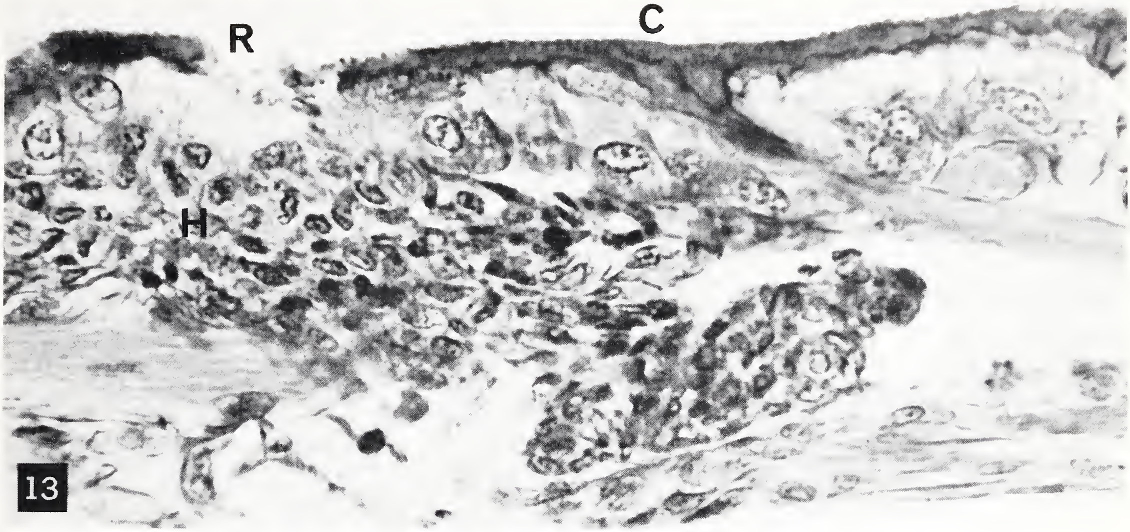

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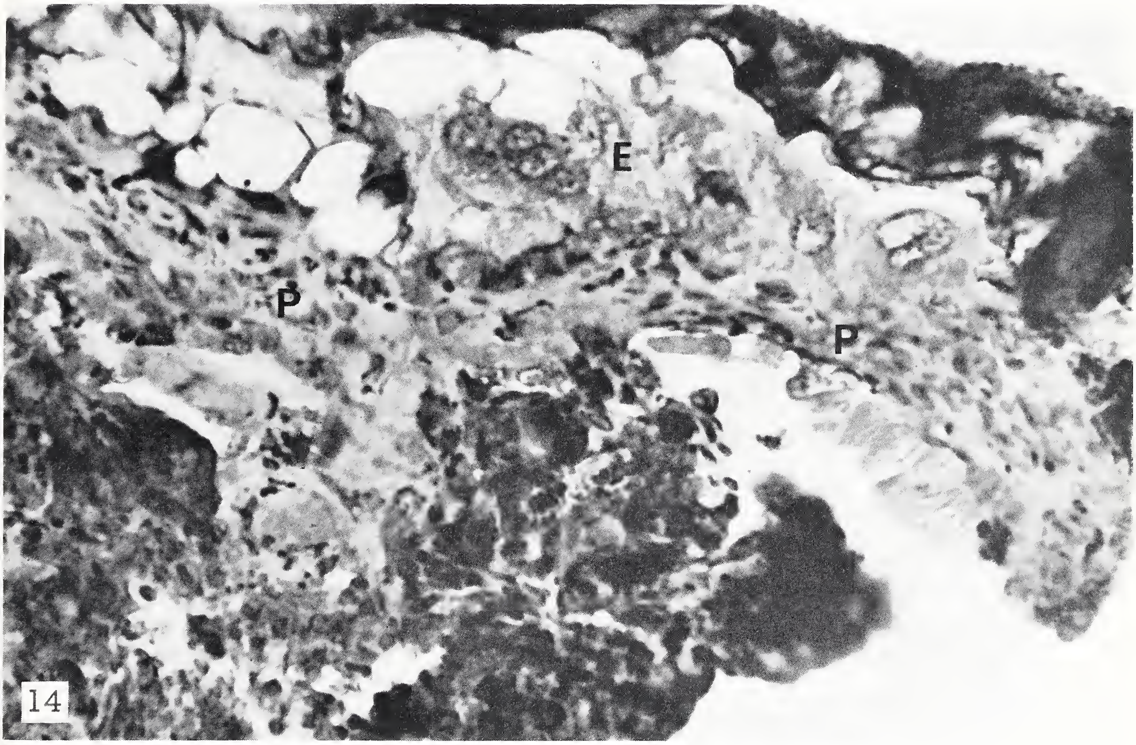

Figure 13.—Time after nerve severance, 7 months. Rupture (R) in epithelium adjacent to circular muscle insertion (C) with hemocytes (H) invading. Hematoxylin and eosin. × 630

Figure 14.—Time after nerve severance, 3 months. Large mass protruding into hemolymph from crop wall. Small, densely basophilic particles (P) appear and the epithelium (E) is degenerating. Hematoxylin and eosin. × 630

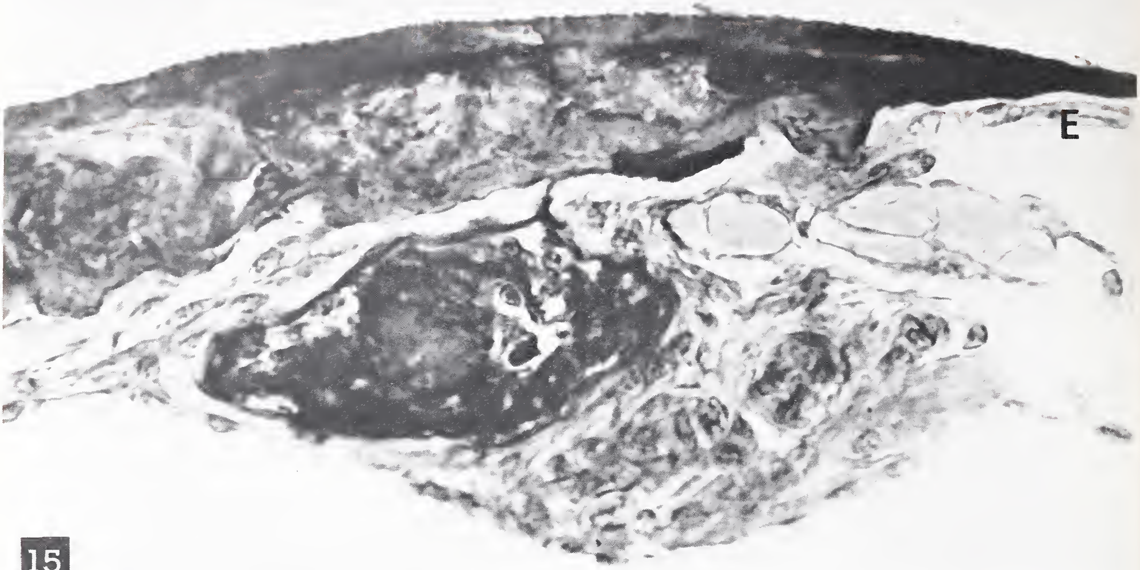

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FIGURE 15.—Time after nerve severance, 5 months. Epithelial cells (E) scant or lacking. Hematoxylin and esoin. × 630

FIGURE 16.—Time after nerve severance, 5 months. Multiple tumors (*arrows*) in crop wall. Crop lumen (F). Hematoxylin and eosin. × 60

Formation of Tumor-Like Lesions in the Cockroach *Leucophaea maderae* After Decapitation[1]

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SUMMARY

Decapitation was shown to be an effective and simple means of inducing tumor-like lesions in the Madeira cockroach, *Leucophaea maderae*. Although lesions developed in most internal organs, significant increases in lesion incidence occurred primarily in the salivary glands and hindgut. The lesions of the salivary glands appeared very similar to those induced in other cockroaches by investigators ligating the salivary ducts. Therefore, it is probable that salivary duct ligation, which is one of the results of decapitation, is also responsible for the induction of the salivary gland lesions in the headless roaches. It is speculated that the hindgut lesions may have resulted from the action of oxyuroid nematodes, which normally live in the hindgut of *L. maderae*. They may have been stimulated to feed on the hindgut wall by the induced starvation. Alternatively, the hindgut may have suffered biochemical injury in its attempts at water conservation; decapitated roaches readily dehydrate, placing a heavy burden on the water retention mechanisms. Other factors that cannot be excluded as possibly being involved in the induction of the various lesions are desiccation, hormone imbalance, starvation, and nerve severance. A yellow-to-brown pigmentation is associated with many of the lesions and with the gut cuticle next to the lesions. The nature of this pigment and its possible function in the lesions are briefly discussed.—Nat Cancer Inst Monogr 31: 419–432, 1969.

TUMOR-LIKE LESIONS have been induced in cockroaches by severance of the recurrent nerve [for review *see* (*1*, *2*)] and by ligation and severance of the salivary ducts (*3–5*). These surgical procedures are very delicate, requiring deftness and patience on the part of the "surgeon." After attempting a few such operations, one cannot help but have great respect for persons who perform such surgical procedures by the hundreds. In my frustration after early unsuccessful attempts to duplicate these

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[1] This investigation was supported by Public Health Service research grant 5358 (to E. A. Steinhaus) from the National Institute of Allergy and Infectious Diseases.

[2] I wish to express my sincere appreciation to Miss Paula Butler, Mrs. Laurel Lamb, and Mr. William Freckleton for their technical assistance, and to Drs. Edward A. Steinhaus, Phyllis T. Johnson, and Albert C. Smith for their critical reading of the manuscript.

experiments, it occurred to me that I could sever both the recurrent nerve and the salivary duct by decapitating the cockroaches. Much to my surprise the headless roaches lived up to 71 days and developed tumor-like lesions in all the organs in which lesions normally develop after nerve severance and salivary duct ligation. This paper describes and discusses the nature of these lesions.

MATERIALS AND METHODS

We decapitated 50 Madeira cockroaches, *Leucophaea maderae*, that had been adults for 7–9 months, by first ligating their necks with surgical thread and then severing the neck anterior to the ligature. The stump was sealed with a drop of the tissue adhesive, methyl-2-cyanoacrylate monomer (*6*). Only females were used, since earlier experiments showed they were more resistant than males to starvation (*7*). The roaches were maintained at a temperature of 26–27°C and at a relative humidity of 60–75%. They were examined periodically, and all those dying or dead were dissected and observed for abnormalities. Abnormal tissues were excised, fixed in Zenker's fluid, embedded in Paraplast, sectioned at 6–8 μ, and stained with hematoxylin and eosin.

RESULTS

At 9 days post decapitation, the first death occurred, and the number of survivors decreased gradually until all the roaches were dead at 71 days post decapitation. Of the 50 roaches, 13 were found heavily infected with the bacterium *Serratia* and too necrotic for dissection; another 4 were too desiccated for examination. One of these latter roaches had a yellow fungus growing out of the intersegmental membranes.

Lesions of various descriptions were found in 19 of the 33 roaches which could be examined when moribund or dead. Only 16 (48%) contained lesions which might be considered tumor-like. It should be noted that lesions were present as early as 9 days post decapitation, and some roaches harbored more than one type of lesion. The lesions were found in the esophagus, crop, salivary glands, salivary reservoir, midgut, hindgut, ovaries, malpighian tubules, and fat body. Those of the malpighian tubules and fat body are common in normal, untreated cockroaches (*2*) and are not considered tumor-like.

Esophageal Lesions

Lesions of the esophagus were found in 3 cockroaches. In one, the entire esophagus grossly appeared tumor-like but was too necrotic to undergo histological processing and so was discarded. The esophageal lesions on the other 2 cockroaches were enough alike histologically to be discussed together. These lesions extended from the cuticular lining of the esophagus through the tunica muscularis and protruded into the hemocoel (figs. 1–4). It was impossible to discern definitely the origin of the cells involved; they appeared to be hyperplastic epithelial cells, though they might have been infiltrating hemocytes. The epithelial cells from surrounding normal areas merged almost imperceptibly with the cells of the lesion, though the latter cells differed from the normal epithelial cells in that their cytoplasm was more eosinophilic and their shape, including that of the nuclei, was perhaps less regular. Some of the cells were spindle shaped and in whorls, a characteristic configuration of blood cells in inflammatory reactions. The cell membranes were quite distinct, and no mitoses were observed. These lesions of the esophagus were tracheated.

Crop Lesions

Although crop lesions were observed in 3 cockroaches, one was in a dead roach and too necrotic for histological procedures. A second crop lesion was essentially identical to the two already described from the esophagus and is not described here. The remaining crop lesion was considerably different and involved an unidentified fungus. It consisted, in part, of a large mass of cells situated next to the crop and salivary reservoir (fig. 5). The nuclei of these cells were heavily stained, small, and round, and appeared not too dissimilar to nuclei in coagulated blood except that here they were situated closer together. They were possibly the pyknotic nuclei of dying blood cells. This mass of cells, as well as the adjacent thoracic muscles, was extensively permeated by fungal hyphae (figs. 6 and 7). In some places, the epithelial cells and muscularis of the crop were completely replaced by blood cells (figs. 8 and 9). The crop cuticle in these areas where there was contact with blood cells was abnormally darkened (amber). Apparently, the blood cells were present in response to fungus infection.

Salivary Reservoir Lesion

A lesion of the salivary reservoir was found in one dead roach but was too necrotic to undergo histological processing. Grossly, it appeared similar to the salivary reservoir lesions in roaches with the recurrent nerve severed (*2*).

Salivary Gland Lesions

Lesions of the salivary glands were found in 3 cockroaches which were decapitated 20–33 days previously (figs. 10–13). Blood cells infiltrated the salivary glands and in one case completely replaced the normal cellular architecture. The blood cells formed "whorls" or encapsulations, sometimes around seemingly healthy blood cells, sometimes around solid cores of amber-colored material [Sutherland's "inclusion cyst material" (*3*, *4*) and Edwards *et al.* "brown inclusions" (*5*)], and other times around rings of amber-colored material which enclosed necrotic cells with pyknotic nuclei. A possible mechanism for the induction of salivary gland lesions will be discussed later.

Midgut Lesions

A lesion was found in the midgut of one cockroach but the tissue was too necrotic to fix properly.

Hindgut Lesions

After the roaches were decapitated, hindgut lesions were most common, being found in 6 roaches. The hindgut of one roach had 2 lesions which histologically appeared almost identical. In cross section, a mass of hardened, amber-colored material was seen between the epithelial cells and the cuticle of the hindgut (fig. 14). The cuticle in contact with the mass of material was abnormally darkened. A single layer of spindle-shaped blood cells separated the amber-colored mass from the epithelial cells, which were flattened, apparently from the pressure of the overlying abnormal mass. No new cuticle was seen between the epithelium and the abnormal mass, as would be expected if this were a healed lesion.

Another hindgut lesion was one of the most massive and unique lesions I have found in *L. maderae* to date (figs. 15–20). Blood cells were virtually nonexistent in this lesion. The epithelial cells were hypertrophic and hyperplastic, and at the periphery of the lesion (but still within it) the nuclei were situated at the lumen side of the cells (fig. 16). The nuclei were generally larger, less dense, and more irregularly shaped than normal nuclei. Most epithelial cells contained pockets of amber-colored granules (fig. 17), and from their lumen side they appeared to be secreting material which varied from eosinophilic-to-basophilic-to-amber colored. Although it appears that this material was primarily of secretory origin, some pyknotic nuclei were incorporated in it. As blood cells were not involved in the lesion, the nuclei probably belonged to dead epithelial cells. The lesion was tracheated (fig. 20), and mitoses were not seen. Possible mechanisms for the induction of hindgut lesions will be discussed later.

Ovarian Lesions

Ovarian lesions were found in 3 cockroaches. Unfortunately, the tissues had been dead up to 24 hours when the lesions were discovered and, though sections were prepared, necrosis had progressed too far for the precise nature of the lesions to be ascertained. It did appear, however, that the fat body adhering to the ovaries had been infiltrated by hemocytes, and in one case the follicular epithelial cells appeared hyperplastic.

Fat-Body Lesions

Fat-body lesions were found in at least 2 roaches. These lesions were identical to those described as resulting from severance of the recurrent nerve (*2*).

Malpighian Tubule Lesions

Although malpighian tubule lesions occurred in unoperated animals, their incidence was higher, and more tubules were affected in experimental animals. In one roach, essentially all of the tubules were hardened and darkened (amber). Their normal cellular architecture had been completely replaced by encapsulating blood cells (figs. 21–23). In cross section, "whorls" of spindle-shaped blood cells surrounded a core of eosinophilic material (necrotic malpighian tubules and blood cells) containing pyknotic nuclei. Often the peripheral regions of this core, *i.e.*, those in contact with the living, encapsulating hemocytes, had hardened and darkened (amber). Sometimes the entire core was amber colored. The animal from which these pathological malpighian tubules were removed may have died from excretory failure; the tubules surely could not have been functioning enough to sustain the animal's life. These lesions were identical to the malpighian tubule lesions in animals with their recurrent nerves severed (*2*).

DISCUSSION

The lesions following decapitation were most easily interpreted as foci of inflammation and repair. The evidence does not demand, or even strongly suggest, that the lesions be interpreted as having resulted from a neoplastic transformation. What then is the possible relationship between decapitation and the development of the inflammatory lesions? First of all, if we compare the individual incidences of lesions resulting from decapitation with those occurring naturally in untreated *L. maderae* (*2*), we see that only two classes of lesions have increased significantly—those of the salivary glands and those of the hindgut.

The lesions in the salivary glands appeared very similar to those described by Sutherland (*3*, *4*) and Edwards *et al.* (*5*), which they induced by ligation and by severance of the salivary duct. Sutherland used the American cockroach, *Periplaneta americana*, in his experiments, and Edwards *et al.* used the same cockroach plus two others, *Blabera gigantea* and *Gramphodorina portentosa.* Although decapitation involves various factors—*e.g.*, ligation of the salivary ducts, severance of the recurrent nerve, induction of a nutritionally deficient state, removal of the influence of the hormonal centers in the head, etc., each of which may play a role in the induction of *L. maderae* lesions—I believe that the salivary gland lesions resulted primarily from the ligature of the salivary ducts. My observations support the contention of Edwards *et al.* that the formation of the lesions is a reactive process rather than neoplastic, *i.e.*, the lesions are not tumors but hemocytic encapsulations "about foci of cell and salivary secretions which are degenerating in response to impounding by ligature." The lesions have the form of typical encapsulation reactions to foreign material (*8*).

The increased incidence of hindgut lesions in experimental animals as compared to unoperated animals might be related to the presence of the oxyuroid nematode *Leidynema appendiculata* in the hindgut of *L. maderae* (*9*). The starvation conditions following decapitation may have resulted in the nematode's attempting to feed on the wall of the hindgut, thereby injuring the wall and eliciting hemocytic infiltration. Taylor (*9*) showed that these nematodes can injure the wall of the hindgut. This explanation would not apply to the one hindgut lesion in which hemocytes apparently were not involved. Alternatively, the hindgut tissue may have been injured in its attempts to maintain a proper water balance in the hemocoel. The tissues of most decapitated animals appeared abnormally dry on dissection. A drop of insect Ringer's solution on the lifeless-appearing heart and gut frequently initiated heart beat and peristalsis, respectively. Undoubtedly, the hindgut in its role as "water conservationist" had to work "overtime," possibly resulting in biochemical injury. Desiccation may also have played a role in the development of lesions in the other tissues.

Insects have been decapitated by various workers. Nishiitsutsuji-Uwo and Pittendrigh (*10*) decapitated a series of roaches, *P. americana*, and noted development of "tumors" in the salivary complex and foregut. They attributed the development of the lesions to severance of the recurrent nerve. In the case of the salivary complex lesions, however, I believe that salivary duct ligature plays the more important role. Schlumberger (*11*) decapitated a series of *P. americana* and showed that there was no change in the response of the hemocytes to implanted foreign material. He noted no "tumor" induction, though maximum survival in his experiments was only 7 days. (Perhaps it should be noted that the same roach, *P. americana*, lived up to 17 days in the experiments of Nishiitsutsuji-Uwo and Pittendrigh.) In my experiments, headless *L. maderae* survived up to 71 days. The incidence of tumor-like lesions reported in these experiments would probably have been greater than the observed 48% had the roaches not died so soon from desiccation.

Ligation of the neck without head removal is a form of decapitation used by various investigators. Burdette (*12*) ligated a "tumorous" strain of *Drosophila* larvae behind the head and noted an increased incidence of "tumors." He believed this result due to blockage of the posterior passage of metamorphosis hormones. Harshbarger and Moore (*13*) similarly ligated larvae of the greater wax moth, *Galleria mellonella*, and noted the development of fat-body lesions not unlike the *L. maderae* lesions. On the basis of additional work (*1*), Harshbarger believes that an upset in the normal hormonal balance is responsible for the lesions. Hormonal imbalance may also have played a role in the induction of lesions in headless *L. maderae*.

Decapitations have been performed in connection with other studies, *e.g.*, behavior studies (*14*) and learning studies (*15*), though no comments were made relative to "tumor" induction.

A common feature of the lesions discussed in this and earlier papers (*2*, *9*) is the yellow-to-brown (amber) pigment, probably melanin. The pigment occurs primarily in necrotic blood cells and in necrotic tissues encapsulated by blood cells. In the early stages of necrosis, the cells become increasingly eosinophilic, and the nuclei become pyknotic. Karyolysis begins, and the cytoplasm loses its eosinophilia and becomes refractory to stains. Nuclei and cell membranes then disappear as amber-colored granules of various sizes and shapes appear in the cytoplasm. The fully necrotic cells appear as dark-amber, hyaline material. As noted earlier, cuticle next to the lesions frequently darkens. Such darkening may be explained on the basis of a diffusible substance, or substances, involved in the melanization process entering the cuticle. Although the gut cuticle does not darken in normal insects, it may contain the enzymes (phenolases) or proenzymes (prophenolases) and activators necessary for darkening (*16*); darkening could then result from an influx of substrate (phenols) from the wound tissue. Or, perhaps both enzyme and substrate molecules diffuse into the cuticle from the lesion.

The darkened areas of the lesions have hardened and are difficult to section. The coloration and hardness are similar to those of normally sclerotized and tanned exocuticle, and perhaps result from a similar process. From a reading of the literature, one cannot help but notice that pigmentation is commonly associated with insect "tumors" and with lesions resulting from wounding of the insect's tissues or from the introduction of parasites or other foreign bodies into the insect. The pigmentation and its concomitant hardness appear to function in the isolation of potentially harmful materials (parasites, microorganisms, necrotic cells, etc.) and, therefore, to be part of a hemocytic defense reaction to diverse abnormal stimuli.

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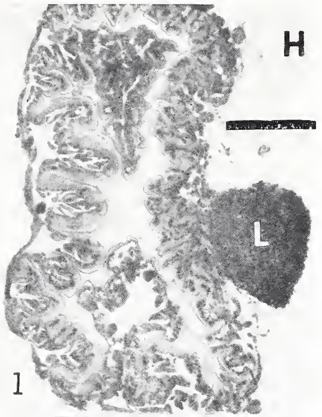
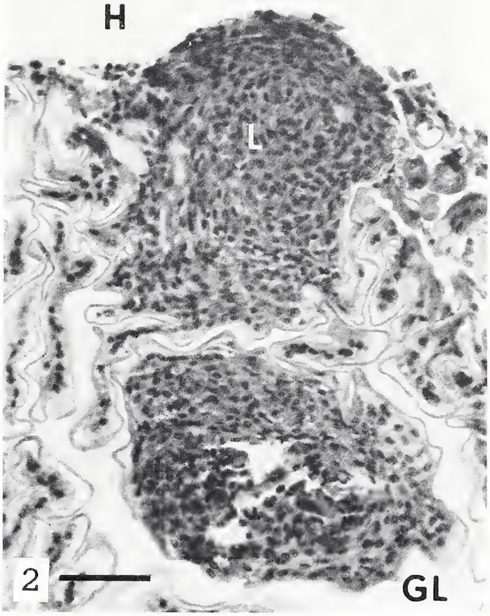
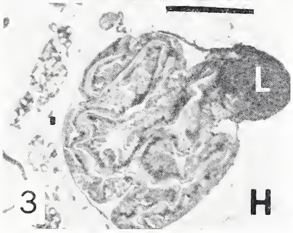
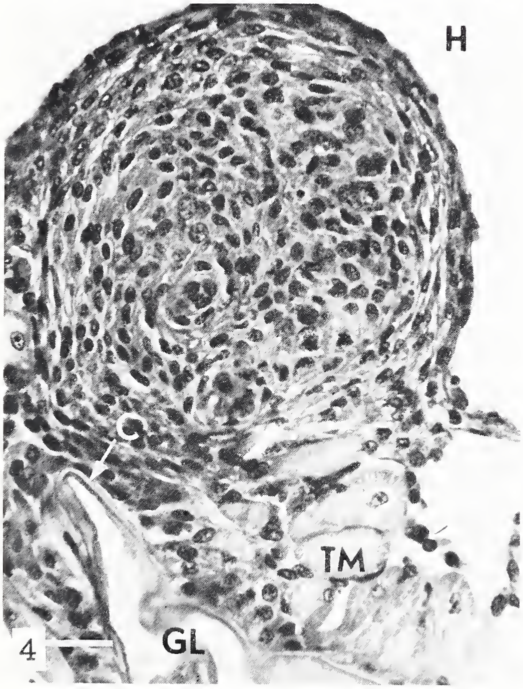
Key: C, cuticular lining of gut; CR, crop; E, normal epithelium; F, fungus; GL, gut lumen; H, hemocoel; HE, hemocytes; L, lesion; SR salivary reservoir; TM, *tunica muscularis*; TR, trachea









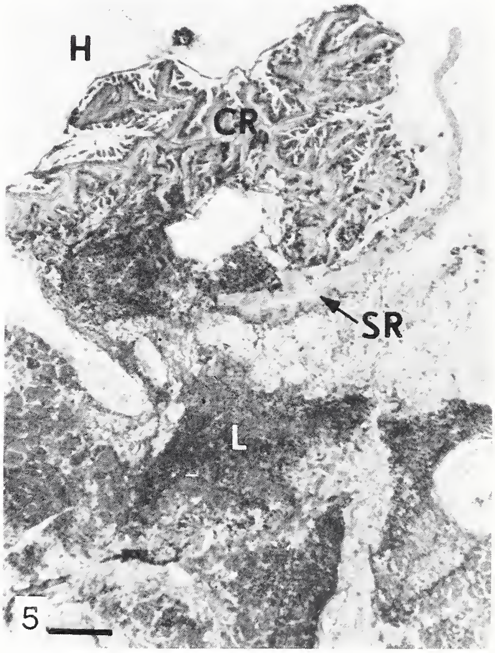


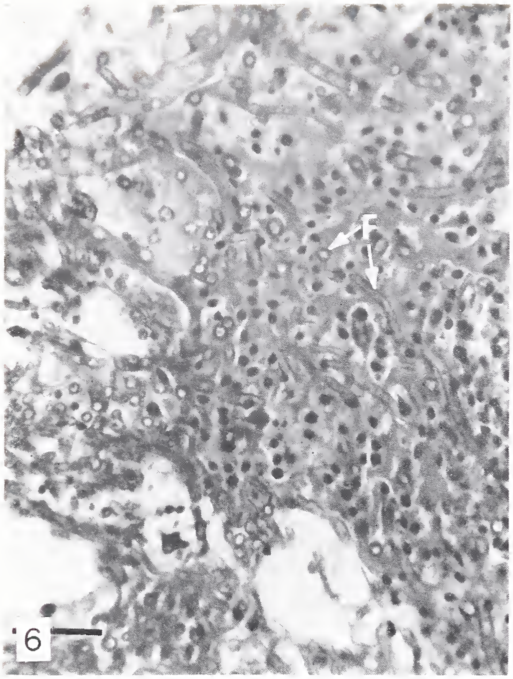
FIGURE 1.—Cross section through esophagus and adhering lesion; 9 days post decapitation. Scale, 240 μ.

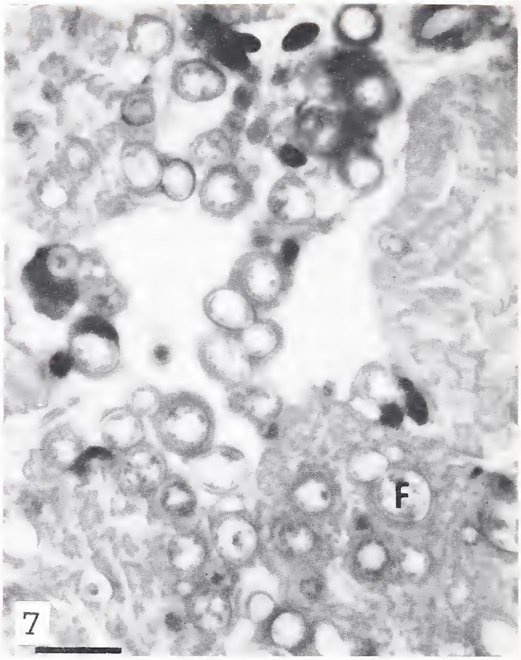
FIGURE 2.—Cross section (at different level) through lesion shown in figure 1. Part of lesion is projecting into gut lumen and is necrotic centrally. *Note* epithelial cells have been replaced but cuticular lining of esophagus is intact. Scale, 80 μ.

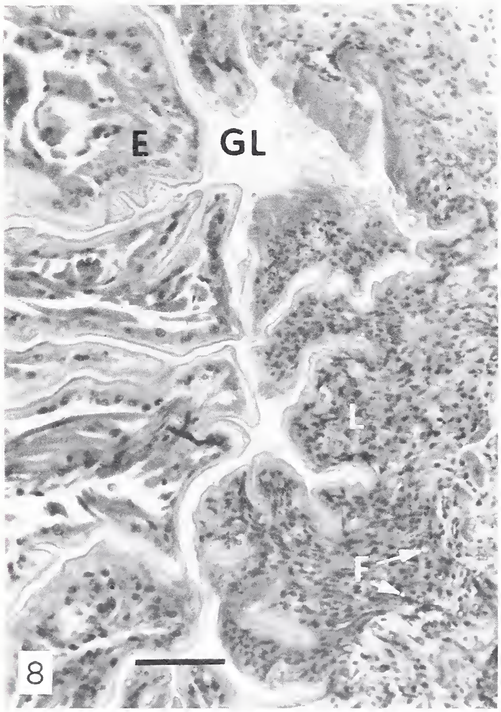
FIGURE 3.—Cross section through esophagus (collapsed in handling) and adhering lesion; 31 days post decapitation. Scale, 240 μ.

FIGURE 4.—Lesion shown in figure 3 at higher magnification. Scale, 40 μ.

FIGURE 5.—Cross section through crop and salivary reservoir (both collapsed in handling) and adhering lesion; 12 days post decapitation. Scale, 240 μ.







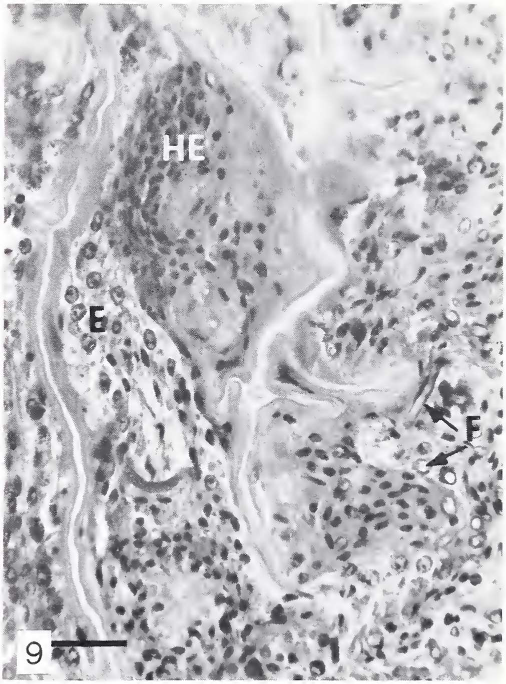
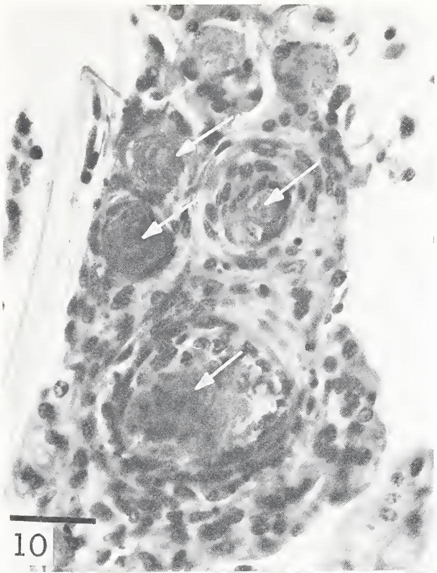


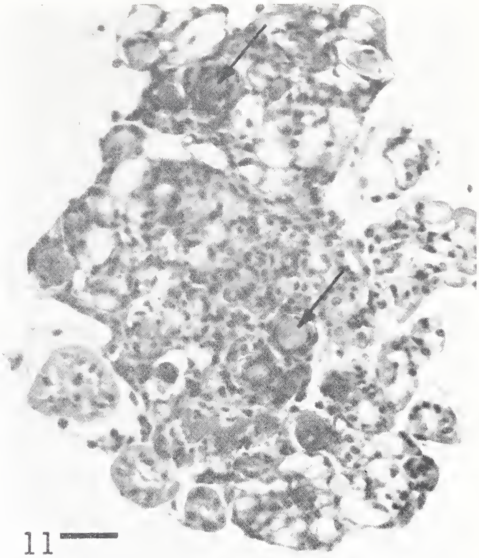
FIGURE 6.—Section through lesion in figure 5 showing numerous fungal hyphae in cross and longitudinal section. Hemocytes have an appearance similar to coagulated blood. Scale, 40 μ.

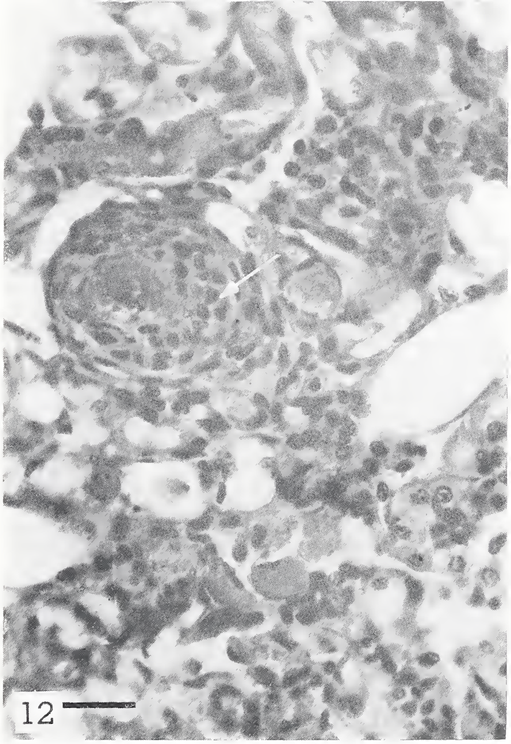
FIGURE 7.—Lesion shown in figure 5 at higher magnification showing fungal hyphae permeating thoracic musculature. Scale, 10 μ.

FIGURE 8.—Crop lesion shown in figure 5 at higher magnification. *Note* that hemocytes have replaced epithelium and much of the *tunica muscularis* in *right half* of photograph. Cuticle next to hemocytes, though intact, has darkened. Scale, 80 μ.

FIGURE 9.—Section similar to figure 8 (though at a different level) showing distinction between infiltrating hemocytes and normal epithelial cells. Scale, 40 μ.







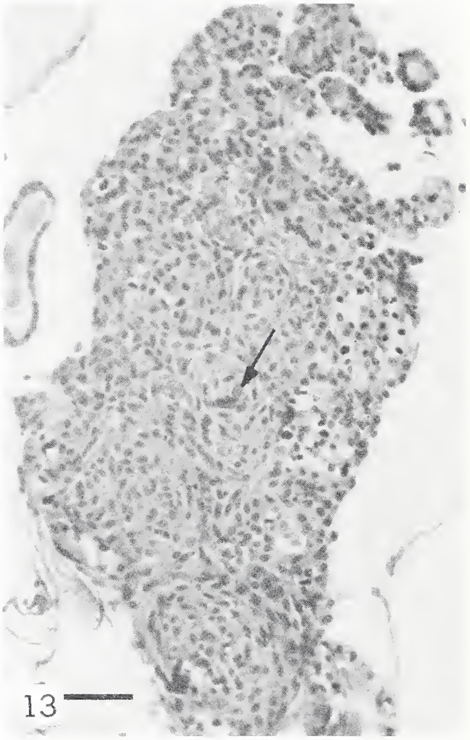


Figure 10.—Section through pathological salivary gland. Infiltrated hemocytes have formed "whorls" or encapsulations around cores of amber-colored material (*arrows*); 28 days post decapitation. Scale, 40 μ.

Figure 11.—Section through pathological salivary gland. *Note* cores of amber-colored material (*arrows*) encapsulated by hemocytes; 31 days post decapitation. Scale, 80 μ.

Figure 12.—Lesion shown in figure 11 at higher magnification and different level. *Note* large hemocytic encapsulation of amber-colored material (*arrow*). Scale, 40 μ.

Figure 13.—Section through salivary gland heavily infiltrated with hemocytes, many of which are in "whorl" formation. Relatively few such encapsulations enclose amber-colored material (*arrow*); 33 days post decapitation. Scale, 80 μ.

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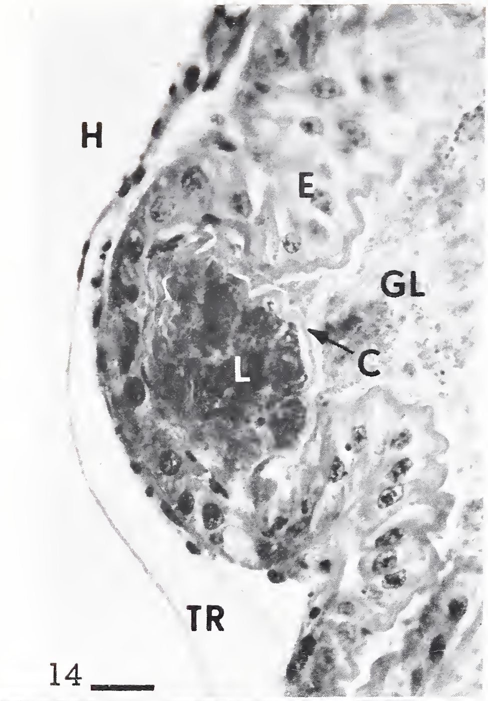
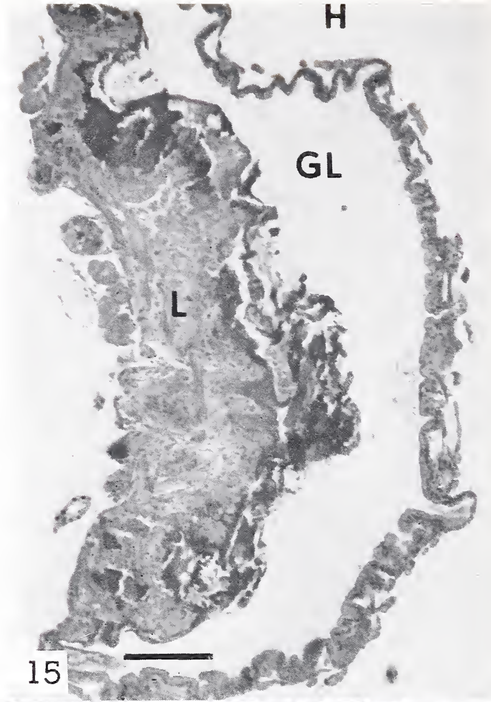
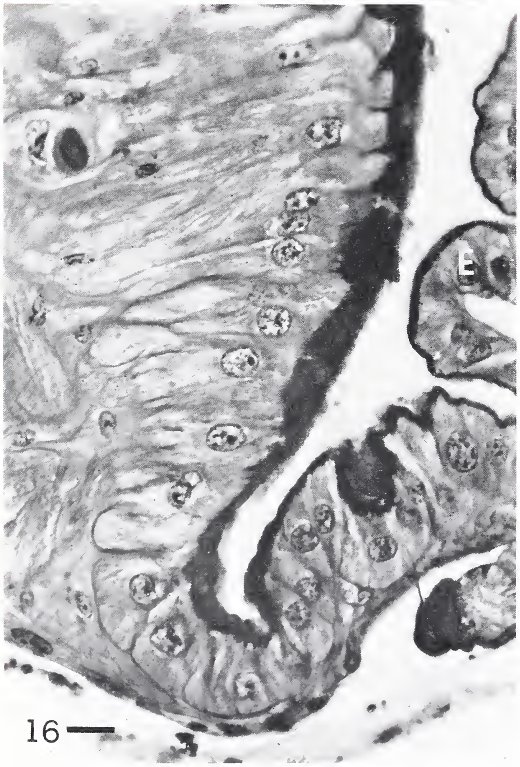






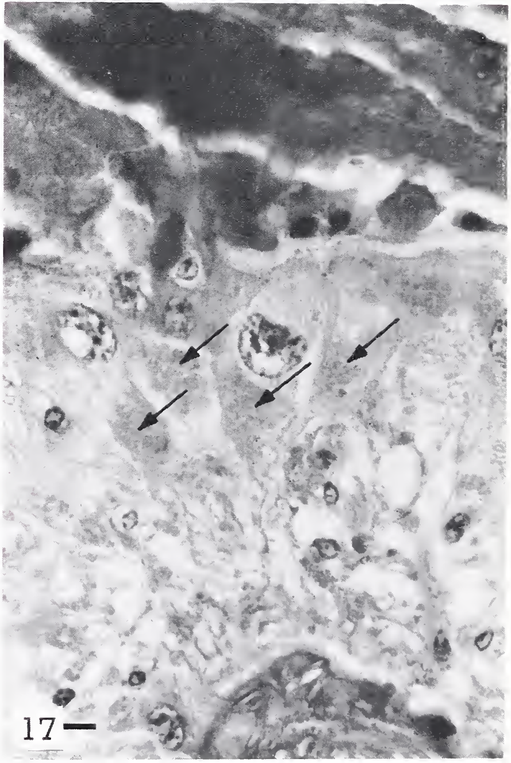
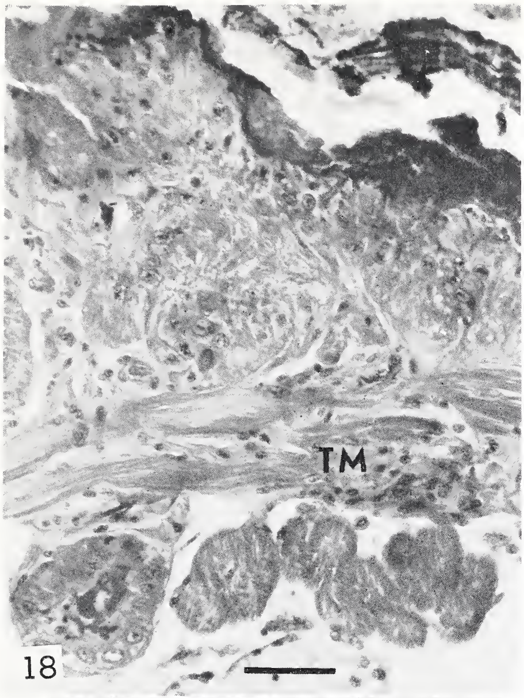


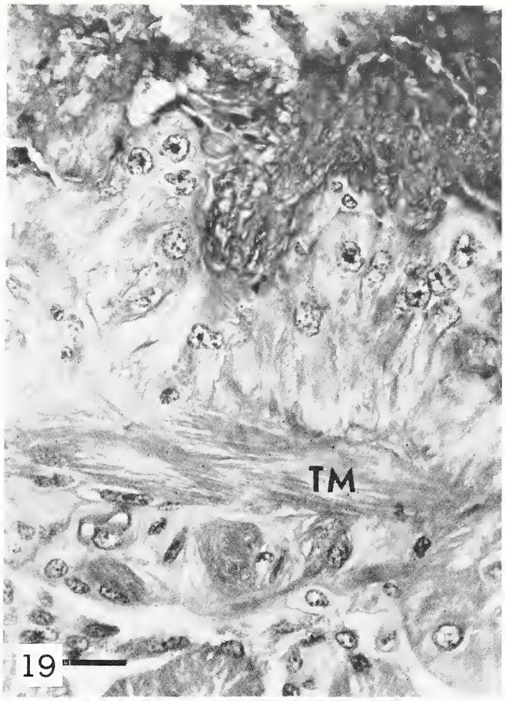
FIGURE 14.—Section through hindgut lesion. Cuticle, though intact, is abnormally darkened over surface of lesion; 28 days post decapitation. Scale, 40 μ.

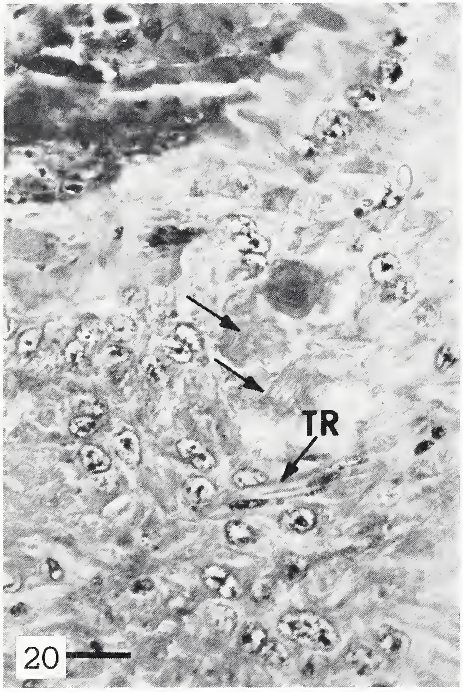
FIGURE 15.—Cross section through hindgut with massive lesion (at *left* in photograph); 47 days post decapitation. Scale, 240 μ.

FIGURE 16.—Periphery of lesion shown in figure 15, though at higher magnification and different level. Normal hindgut epithelium is seen at *right*, and greatly enlarged epithelium of lesion at *left*. Scale, 40 μ.

FIGURE 17.—Lesions shown in figure 15 at higher magnification. *Note* amber-colored granules (*arrows*) within abnormal epithelial cells, nuclei of diverse sizes and shapes, and secreted material (*top third* of photograph). Scale, 20 μ.







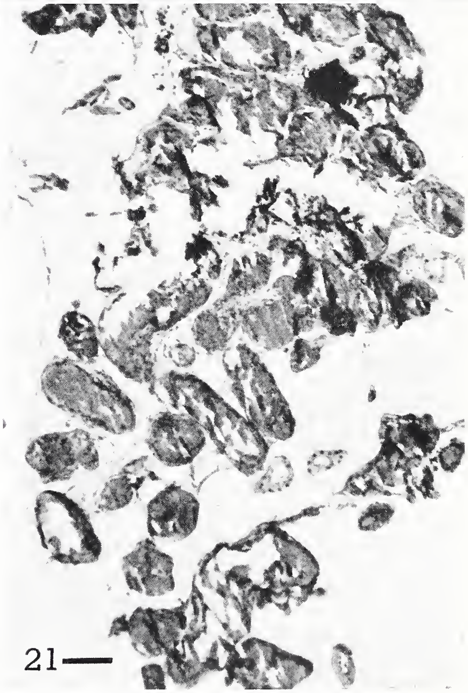
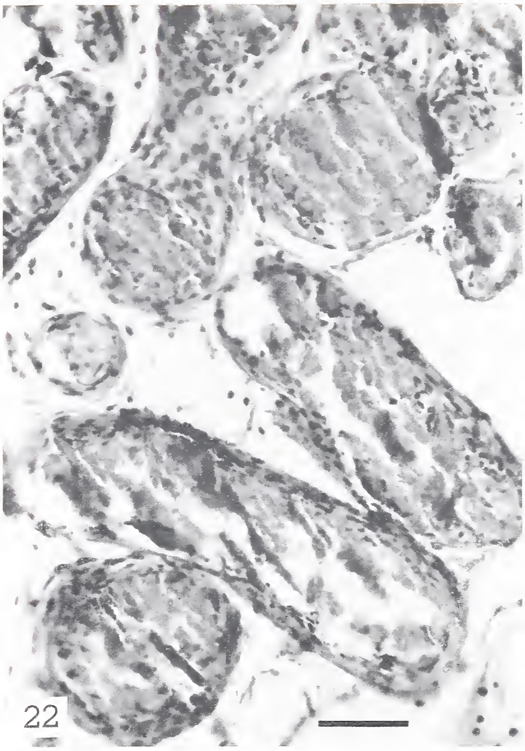


FIGURE 18.—Lesions seen in figure 15 showing, *from bottom up*, the relatively normal *tunica muscularis*, the abnormal epithelium, and the secreted material. The latter varies from eosinophilic to basophilic, to amber-colored. Scale, 80 μ.

FIGURE 19.—Another view of lesion seen in figure 15. *See* legend to figure 18. Scale, 40 μ.

FIGURE 20.—Another view of lesion seen in figure 15. *Note* tracheole, myelin-like, amber-colored figures (*arrows*), atypical epithelial cells, and secreted material (*top left*). Scale, 40 μ.

FIGURE 21.—Section through mass of abnormal malpighian tubules; 21 days post decapitation. Scale, 240 μ.



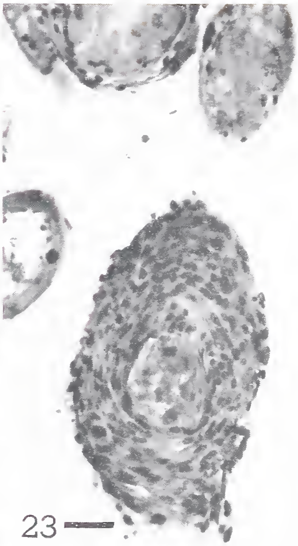


Figure 22.—Lesions shown in figure 21 at higher magnification. *See* text for description of lesions. Scale, 80 μ.

Figure 23.—Encapsulated malpighian tubules. *See* text for description of lesions. Scale, 80 μ.

Effects of Certain Carcinogens on *Periplaneta americana* (L.)[1,2]

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SUMMARY

Nymphs and adults of *Periplaneta americana* (L.) were treated with 3-methylcholanthrene, 2-fluorenamine, or *N,N*-dimethyl-*p*-azoaniline as food supplements, topical applications, or injections. Croton oil was sometimes used as a topical irritant. Incidence of abnormal growths after such treatments generally was less than 20%, and the sites of the growths were not specific for any compound generally. Histologic studies revealed that such growths were wound-healing reactions that differed little from such reactions observed in untreated individuals. Regeneration of the epithelium was controlled by the encapsulating hemocytes, and there was no indication that the compounds were carcinogenic.—Nat Cancer Inst Monogr 31: 433–445, 1969.

EXCEPT FOR *Drosophila* sp., few investigations have dealt with the effect of chemical carcinogens on insects (*1*). Only two researchers (*2, 3*) have used an insect such as the cockroach, having a relatively long lifespan, which would allow for a longer exposure and reaction to treatments and might make a study of possible tumor development less difficult. Schlumberger (*2*) implanted methylcholanthrene in *Periplaneta americana* (L.), observed effects similar to those following talc implantation, and concluded that they were not neoplasms. Noland and Baumann (*3*) reared *Blattella germanica* (L.) on diets containing azo dyes and observed lesions in the Malpighian tubes, which did not appear to be autonomous growths. Wautier and Wautier (*4*) reviewed these and other studies and concluded that compounds capable of inducing malignant tumors in mammals are incapable of provoking similar growths in insects. However, in mammals tumor induction is variable between individuals, species, and treatments (*5*). Since, in previous research with the

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[3] I gratefully acknowledge the valuable assistance of Mrs. J. M. Chillseyzn.

cockroach, hemocyte encapsulation of implanted methylcholanthrene might have prevented circulation of the chemical (*2*), and studies with *B. germanica* (*3*) were not extensive, further investigations were deemed necessary. Therefore, since 1964 our laboratory has investigated the effect on *P. americana* of various types of treatments with several chemical carcinogens. This report summarizes the effects and the histology of abnormal growths observed.

MATERIALS AND METHODS

Insects.—Nymphs and adult *P. americana* from a laboratory colony in culture for over 20 years were used in all experiments. Cockroaches were treated in groups of 10, maintained in plastic containers under a light: dark regimen of 12 hours: 12 hours, and dissected at death or at the end of the experiment.

Chemical carcinogens.—Carcinogens included 3-methylcholanthrene (MCA), 2-fluorenamine (FL), and *N*,*N*-dimethyl-*p*-phenylazoaniline (DPAA). An aged preparation of DPAA (DAB, *p*-dimethylaminoazobenzene) was also used in initial studies.

Diets containing carcinogens.—Diets containing 0.1, 0.2, 0.4, and 0.8% (w/w) were prepared with chloroform as solvent. A volume of 10 ml was sufficient for preparing 50 g of ground Purina laboratory food. After stirring, solvent evaporation, and additional blending, generally the diets were made into pellets (Parr Pellet Press, Parr Instrument Co., Moline, Ill.). A control diet was similarly made with chloroform.

Topical application and injection.—Solutions were applied topically to the ventral, cervical, and prothoracic regions by means of a microsyringe and a Dutky and Fest microinjector, which was calibrated with the solvent. The same apparatus was used for injection into the thoracic region.

Treatments.—The various experiments in which cockroaches were treated with chemical carcinogens were as follows:

Experiment I: First instar nymphs (30 per diet concentration) were supplied the diets *ad libitum* for 7 months, followed by 5 months on untreated food. Mortalities during the experiment and individual weights and adult: nymph ratios at the end of the experiment were recorded.

Experiment II: Last instar nymphs (30 per diet concentration) were supplied the diets *ad libitum* for 7 months followed by 3 months on untreated food. Mortalities were recorded at the end of the 7th and 10th month.

Experiment III: Last instar nymphs (30 per treatment) were subjected to the following treatments: *a*) 3.6 μliter 0.25% (w/v) carcinogens in acetone, 5 weekly topical applications; *b*) 3.6 μliter 0.5% (w/v) carcinogen in chloroform, 4 weekly topical applications; *c*) 3.6 μliter 0.5% (w/v or to saturation) in peanut oil, 5 weekly injections, and *d*) controls treated similarly with solvents. Groups from treatments *a*) and *b*) were dissected

1 week after the last application, those from *c*) 3 weeks after the last injection.

Experiment IV: Young adult females (10 per treatment) were supplied carcinogen diets (0.4%) and after 2 weeks received 2 weekly topical applications of 3.6 μliter croton oil or 50% (v/v) croton oil in acetone. Survivors were examined 2 months after the last application.

Experiment V: Young adult females (20 per treatment) were supplied carcinogen diets (0.4%) and after 3 months received 4 weekly topical applications of 1.6 μliter 50% (v/v) croton oil in acetone. Individuals were examined 2 months after the last application.

Histology.—Abnormal growths observed on dissection were fixed in Carnoy's and stained with Harris' or Ehrlich's hematoxylin and eosin.

RESULTS

Toxicity of Carcinogens and Their Effect on Growth and Development

In experiments I and II the mortalities experienced by groups supplied with carcinogen diets were generally similar to those receiving the control diet. Exceptions to this were slightly higher mortalities of nymphs in experiment I on 0.1–0.8% MCA and 0.8% DAB. Survival of nymphs on 0.1–0.2% FL was slightly higher than all other groups until they were removed from the diet, after which mortality increased to slightly above that of control groups. After 7 months on most carcinogen diets, nymphal weight was approximately 50% that of the control groups, indicating a slower rate of growth and development. These processes were severely retarded in groups receiving 0.8% DPAA, DAB, and MCA, their weights being 23–30% those of the controls. When supplied with untreated food, such groups proceeded to the adult stage.

Repeated topical applications of carcinogens and solvents in experiment III were increasingly toxic to last instar nymphs, largely interfering with successful ecdysis. This was particularly true for chloroform.

Repeated injections of carcinogens in peanut oil were relatively nontoxic. On dissection, hemocyte encapsulated globules of the oil were generally observed at the site of injection.

In experiment IV, the toxicity of topically applied croton oil increased after the second application, and therefore, in experiment V, the dosage was reduced.

The incidence of the nematode, *Leidynema blattae-orientalis* (Hamm), in this colony of *P. americana* is normally over 95%. Diets containing FL, 0.8% MCA, 0.8% DPAA, or 0.8% DAB significantly reduced or eliminated the incidence of this nematode. In experiment I, reinfestation occurred in groups previously supplied FL diets. In experiment II, a reinfestation occurred in all the diets above, except for FL, 0.8%.

Sites and Incidence of Abnormal Growths

The principal (10–20%) and secondary (less than 10%) sites of abnormal growths observed after various treatments are given in tables 1–5. Sites included the salivary glands, crop, proventriculus, gastric coeca, midgut, Malpighian tubes, pylorus (hindgut valve posterior to Malpighian tubes), hindgut, and rectum. Generally, incidence in these tissues was less than 20% and the sites were not specific for carcinogen. In long-term diet studies, the Malpighian tubes were the most common site, with the rectum, hindgut, and crop lesser sites. In topical application and injection studies, the Malpighian tubes and midgut were the most common sites, although acetone itself affected the Malpighian tubes. Similarly, growths were observed in the digestive tracts of individuals topically treated with croton oil.

Histology of Abnormal Growths

Salivary glands.—Abnormal growths of salivary glands consisted of hemocytic encapsulation, destruction of zymogen and parietal cells, and presence of inclusion cyst material. These processes appeared similar to those following duct ligature (*6*).

Crop and proventriculus.—Growths in the crop generally consisted of a thickening of the crop intima and additional subintimal layers which were either products of the epithelial cells or their breakdown. These layers were faintly acidophilic, in contrast to the basophilia observed after nerve lesion (*7*). Layers of hemocytes were affixed to the crop wall, but the point during the above processes when they initiated encapsulation could not be determined. Epithelial repair was observed in one individual. The single proventricular growth (table 2, DAB) consisted of hemocyte encapsulation.

TABLE 1.—Sites of growths in *P. americana* maintained on diets containing carcinogens for 7 months during early nymphal instars

Compound, % concentration		Principal site, 10–20%	Secondary sites, <10%
FL	0.4		MT*
	0.8	Hindgut	Midgut
DPAA	0.1–0.8		Hindgut, pylorus, rectum, MT, midgut
DAB	0.4		MT
	0.8	Hindgut	Midgut
MCA	0.2	MT	
	0.4	MT	
	0.8	Crop	
Control			Rectum

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* Malpighian tubes.

TABLE 2.—Sites of growths in *P. americana* maintained on diets containing carcinogens for 7 months during last nymphal instar and adult stage

Compound, % concentration		Principal site, 10–20%	Secondary sites, <10%
FL	0. 1	Pylorus	MT*
	0. 2		Rectum, MT, midgut, crop, fat body
	0. 4		Rectum, MT, hindgut, pylorus
	0. 8		MT
DPAA	0. 1		Hindgut, crop
	0. 2		Rectum, MT, hindgut, midgut, crop
	0. 4	MT, hindgut, pylorus	Rectum, crop, coeca
	0. 8	MT	Rectum
DAB	0. 1	Rectum	
	0. 2	Pylorus,† rectum	Fat body
	0. 4	MT,† rectum	Salivary glands, crop
	0. 8	MT,† hindgut, rectum	Proventriculus
MCA	0. 1	Rectum	MT, midgut, pylorus
	0. 2	Pylorus	Rectum, MT, pylorus
	0. 4	Crop	Rectum, MT, hindgut, midgut, salivary glands
	0. 8	MT, crop	
Control		None	

* Malpighian tubes.

† Incidence 30–50%.

TABLE 3.—Sites of growths after repeated topical applications or injections of carcinogens

Treatment, solvent, compound*		Principal site, 10–20%	Secondary sites, <10%
Topical application, in acetone	FL	MT†	
	DPAA	MT, hindgut, midgut	Pylorus
	MCA	Pylorus	Midgut, salivary glands
	Solvent	MT	Midgut
Topical application, in chloroform	FL		MT, midgut, pylorus, coeca
	DPAA	MT, midgut	Hindgut
	MCA	MT, midgut	Coeca
	Solvent	Hindgut	MT
	Control	None	None
Injection, in peanut oil	FL		MT, midgut, pylorus, coeca
	DPAA	MT, midgut	Hindgut
	MCA	MT, midgut	Coeca
	Solvent	Hindgut	MT, midgut, coeca
	Control	None	MT

* Concentration in acetone 0.25%, 5 weekly applications; in chloroform 0.5%, 4 weekly applications; in peanut oil 0.5% or to saturation, 5 weekly injections; volume 3.6 μliter.

† Malpighian tubes.

Table 4.—Sites of growths in *P. americana* maintained on carcinogen diets (0.4%), before 2 topical applications of croton oil and/or acetone

Topical application*	Diet	Principal site, 10–20%	Secondary sites, <10%
Acetone	FL		Midgut, hindgut
Croton oil:acetone	FL	Hindgut	Crop
Croton oil	FL	Midgut	Crop
Acetone	DPAA		MT,† midgut
Croton oil:acetone	DPAA		MT, midgut, hindgut
Croton oil	DPAA	Midgut, hindgut	Crop
Acetone	MCA		Hindgut, crop
Croton oil:acetone	MCA		Midgut
Croton oil	MCA		Midgut, crop
Acetone	Control	None	None
Croton oil:acetone	Control	Hindgut	Midgut
Croton oil	Control	Midgut,‡ hindgut	MT

* Diets started 2 weeks before first of 2 weekly applications (3.6 μliter); killed 2 months after second application. Croton oil:acetone, 50% v/v.

† Malpighian tubes.

‡ Incidence over 50%.

Table 5.—Sites of growths in *P. americana* maintained on carcinogen diets (0.4%), before 4 topical applications of croton oil and acetone

Topical application*	Diet	Principal site, 10–20%	Secondary sites, <10%
Acetone	FL	Hindgut	MT,† midgut, crop
Croton oil:acetone	FL		MT, hindgut
Acetone	DPAA		
Croton oil:acetone	DPAA	Crop	MT
Acetone	MCA	MT‡	Hindgut
Croton oil:acetone	MCA	MT‡	Coeca, hindgut
Acetone	Control	None	None
Croton oil:acetone	Control	MT, coeca, hindgut	Midgut, crop

* Diets started 3 months before first of 4 weekly applications (1.6 μliter); killed 2 months after fourth application. Croton oil:acetone, 50% v/v.

† Malpighian tubes.

‡ Incidence over 30%.

Coeca and midgut.—Growths observed in the coeca consisted of excessive exfoliation of cells and groups of cells into the coecal lumen (fig. 1), sometimes filling and possibly plugging the lumen. Generally hemocyte encapsulation did not occur until the epithelium ruptured. At this point hemocytes in large numbers appeared, sealing off the escape of lumen contents. In some cases, it appeared that rupture was sudden, with a few hemocytes forming a large plug of fibrous material, the interspaces of which were filled possibly with hemolymph. Later stages involving necrosis were observed, but no repair was evident. Growths of the midgut also consisted of exfoliation of epithelium (fig. 2) and encapsulation by hemocytes.

Malpighian tubes.—Abnormal growths of the Malpighian tubes generally were small nodules on 1–10 tubes and, in a few individuals fed DPAA or DAB, large, ovoid bodies 1 × 2 mm protruding from the surface of the digestive tract (fig. 3). The latter bodies contained a lumen (fig. 4) that was lined with epithelium derived from one of the tubes encapsulated by the mass of hemocytes (fig. 5). Also within the mass were large cells, the remains of other encapsulated tubes. Clumps of hemocytes in the lumen possibly were part of necrotic material removed by enzymatic digestion before histologic examination.

Changes in the epithelium of the tubes often occurred prior to hemocyte encapsulation (fig. 6). These included increase in nuclear size, nucleolus enlargement, chromatin clumping, mitosis, as well as increase in the thickness of the basement membrane and size of the lumen. In some cases, cells ruptured into the lumen. It was impossible to determine if any of these changes was due to lumen blockage in the proximal parts of the tubes. After encapsulation by hemocytes, many cells of the tube epithelium were destroyed (fig. 7). However, some remained, increased in size, and proliferated nests of cells (fig. 8), possibly eventually yielding a continuous epithelial vesicle as in the large, ovoid bodies (fig. 3). No unlimited epithelial growth was observed. In one instance, a new tube appeared within the original tube (fig. 9).

Pylorus and hindgut.—Abnormal growths in the pylorus and hindgut (fig. 10) were similar to those in the crop. A thick layer of homogeneous material (HM) appeared between the epithelium and the intima, which itself was sometimes thicker. Apparently the epithelium deposited the material that, although homogeneous in texture and noncellular at first, was faintly basophilic or faintly acidophilic in certain areas.

In some cases, hemocytes had invaded the epithelium and, along with epithelial cells, were destroyed, the remains adding to the HM. Other hemocytes appeared in large numbers between the muscles and at the base of the epithelium. Present within such masses were large cells, single or in groups. In some hindguts, these cells gave rise to a new epithelium, and the dead cells and HM were sloughed into the lumen.

Similar growths have been observed in control groups in other studies. The possibility that nematodes irritate the pylorus and hindgut to form these growths is currently being investigated.

Rectum.—Abnormal growths of the rectal pads were similar to the tumefactions described elsewhere (*8*). They involved production of HL, destruction of epithelium, hemocyte encapsulation, and, sometimes, regeneration of the epithelium.

DISCUSSION

Based on the histologic similarities of abnormal growths observed in these studies and their general nonspecific sites and low incidence, it appears that the carcinogens MCA, FL, DPAA, and DAB as used sometimes in combination with croton oil as an irritant, are noncarcinogenic to *P. americana.* Instead, these compounds act as low-level toxicants to most tissues of the digestive tract. Some tissues, such as the Malpighian tubes, appear more susceptible to MCA, DPAA, and DAB, but this same tissue is susceptible to repeated topical applications of acetone.

Generally the response of tissues to the chemical injury of the carcinogens has been one of wound-healing, including the defense mechanism of hemocyte encapsulation and the frequent appearance of large cells, from which a new epithelium can be derived. The reparative capabilities of insect tissues possibly explain the decreased incidence of growths in nymphs in comparison to adults maintained on carcinogen diets (tables 1 and 2); after 7 months on such diets, nymphs were fed normal food for 5 months before examination, while this period for adults was 3 months. One exception to this general healing process has been the midgut epithelium, which as endoderm in origin may not possess reparative capabilities. The formation of a new epithelium within the large nodules observed in cockroaches fed DPAA and DAB is further evidence of the importance of hemocyte encapsulation as a mold to limit the epithelial growth. Possibly under such conditions this epithelium is not exposed to significant amounts of carcinogen. While future studies might concern the possible effect of carcinogens on this actively growing tissue, it might be of greater value to determine the insect metabolism of such compounds and their affinity for insect protein.

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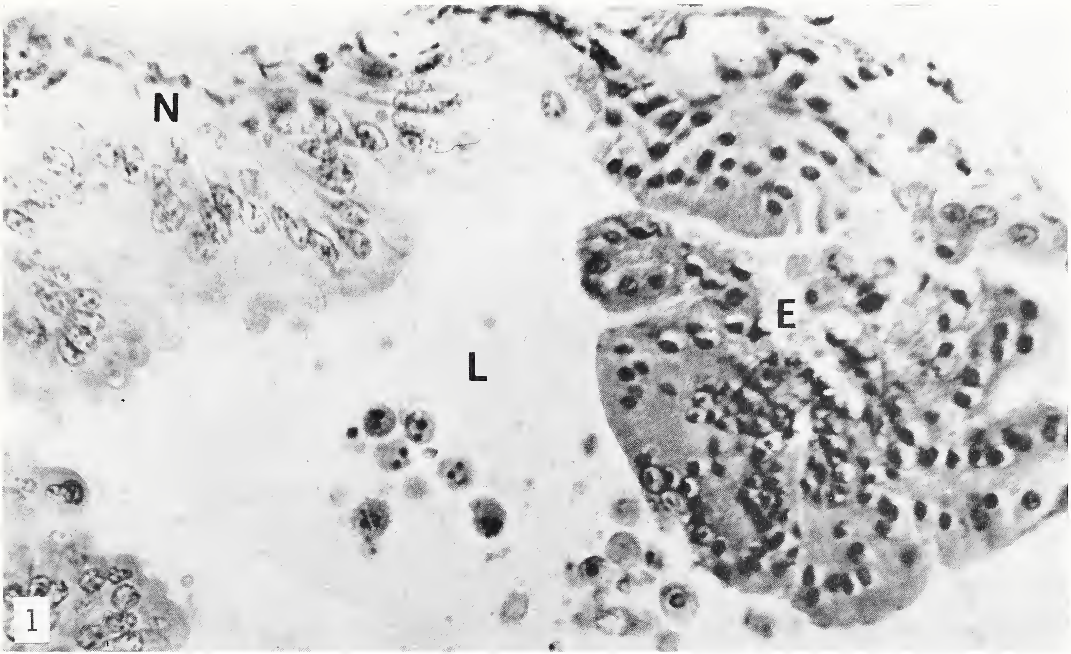

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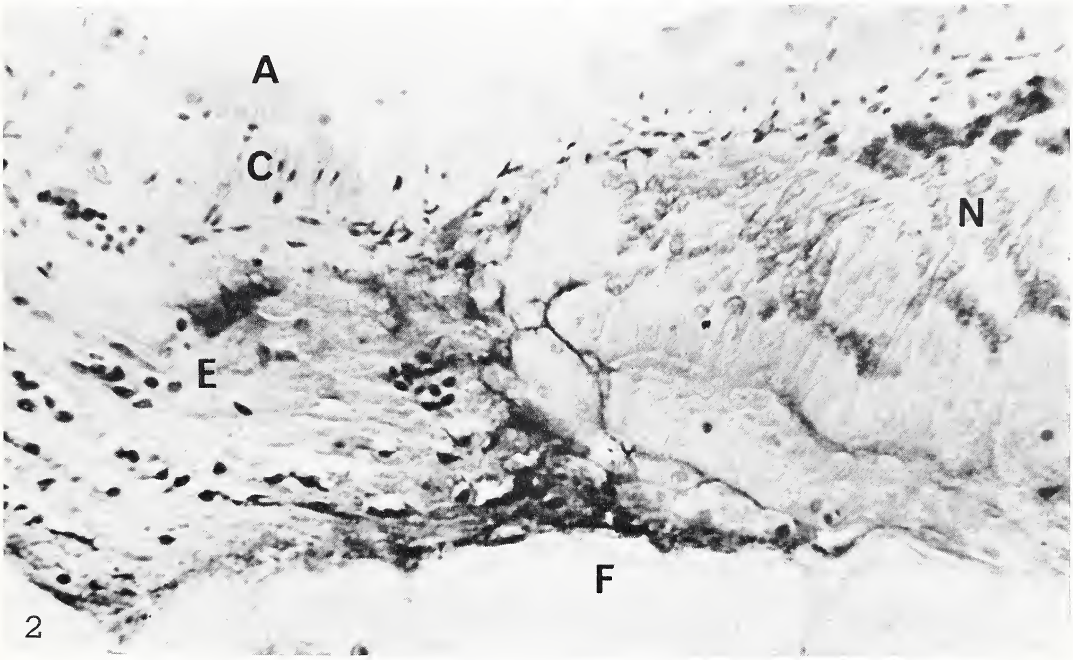

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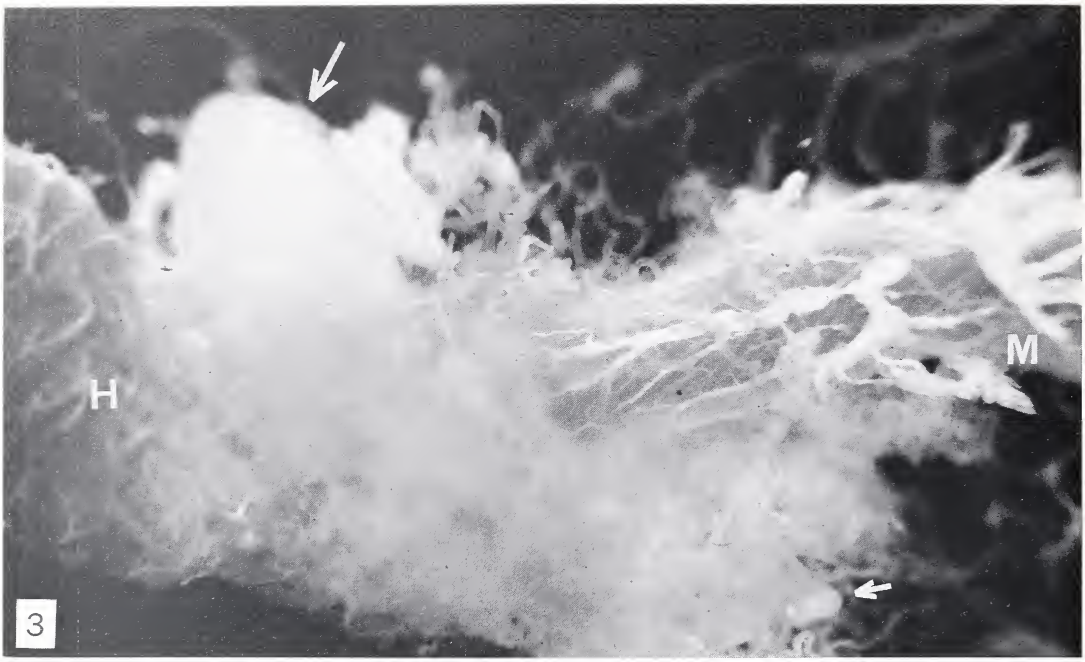
FIGURE 1.—Coecum, cross section, with exfoliation (E) of epithelium into lumen (L); relatively normal epithelium (N). DPAA injection. Hematoxylin and eosin. × 475

FIGURE 2.—Midgut, longitudinal section, with exfoliation (E) into the lumen (F); relatively normal epithelium (N), and layers of longitudinal (A) and circular (C) muscle. DPAA diet. Hematoxylin and eosin. × 475



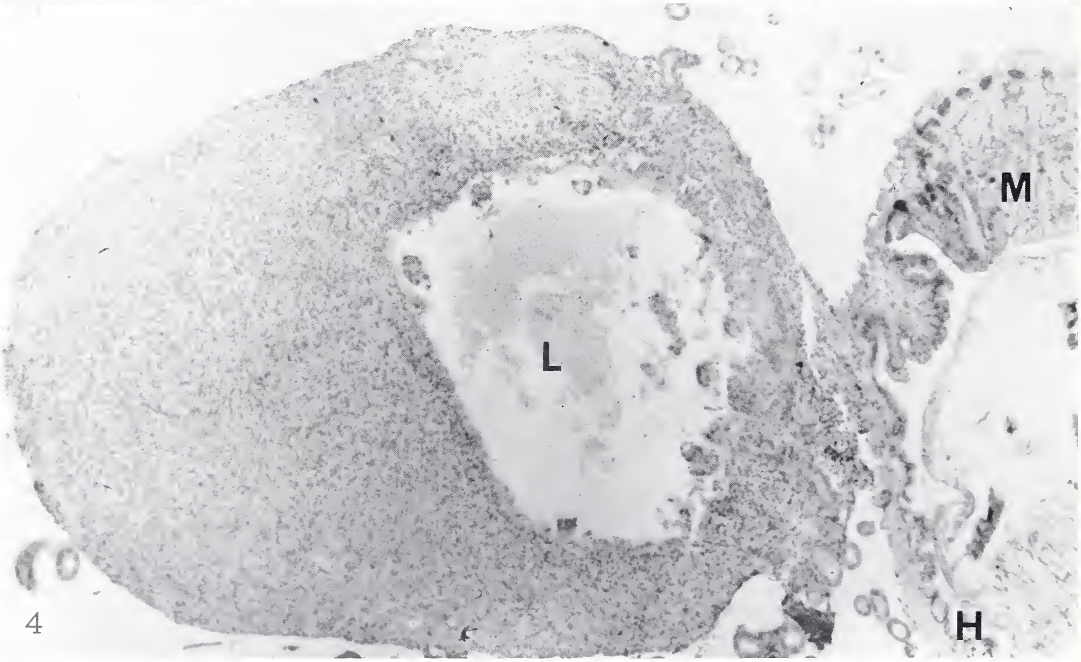


FIGURE 3.—Large ovoid body (*large arrow*) and small nodule (*small arrow*) at junction of the midgut (M) and hindgut (H), unfixed tissue. DAB diet. × 28

FIGURE 4.—Large, ovoid body of figure 3, with lumen (L) and attachment to junction of midgut (M) and hindgut (H). Hematoxylin and eosin. × 75

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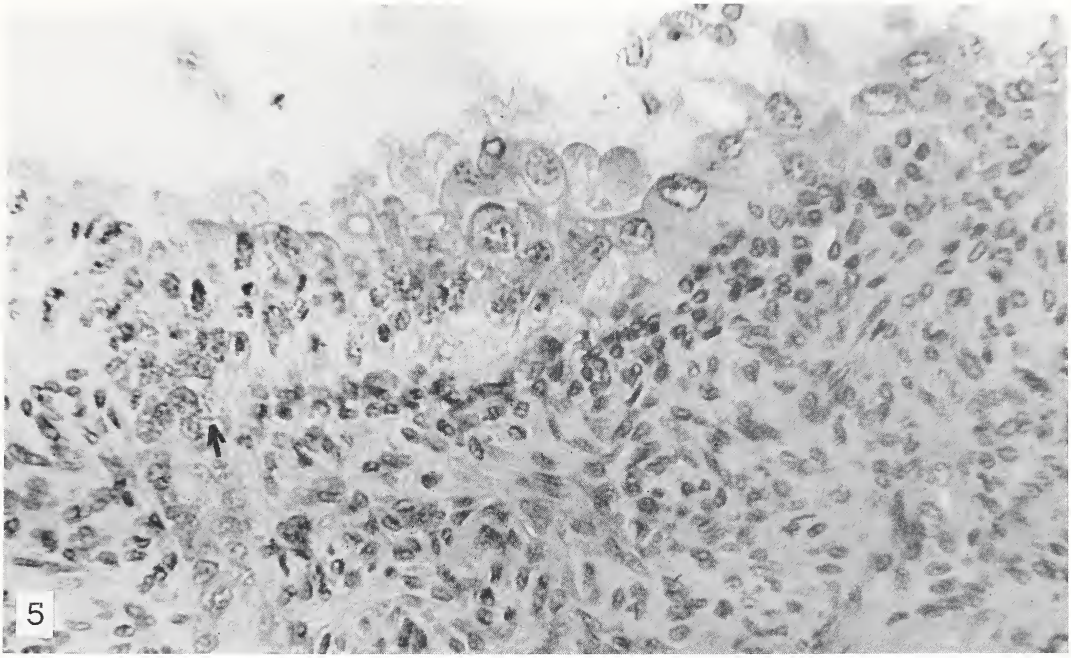

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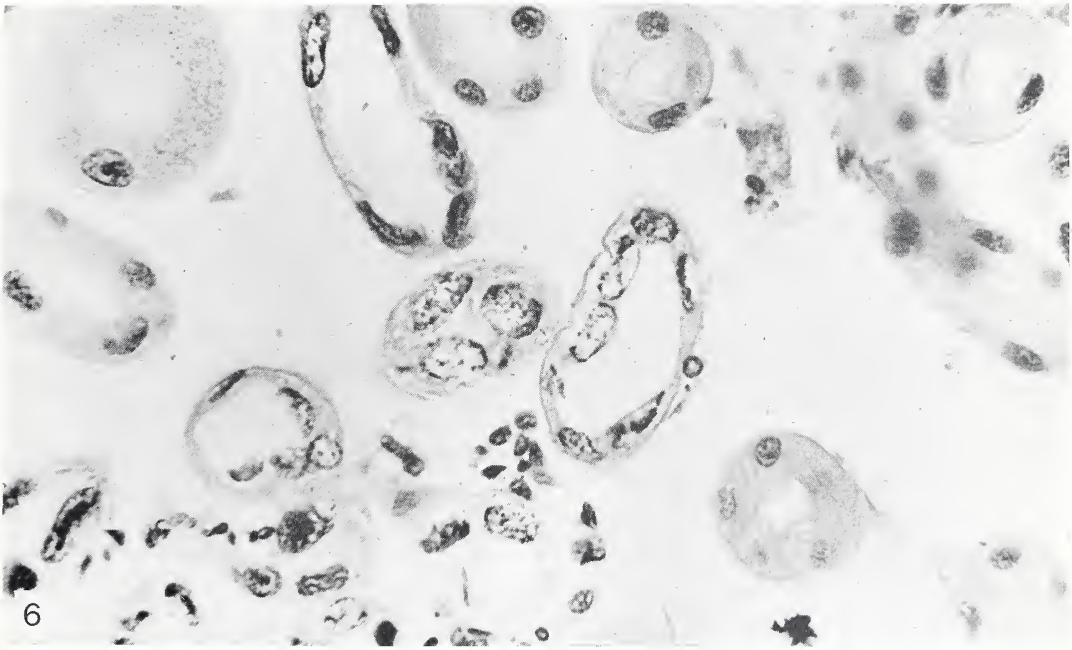
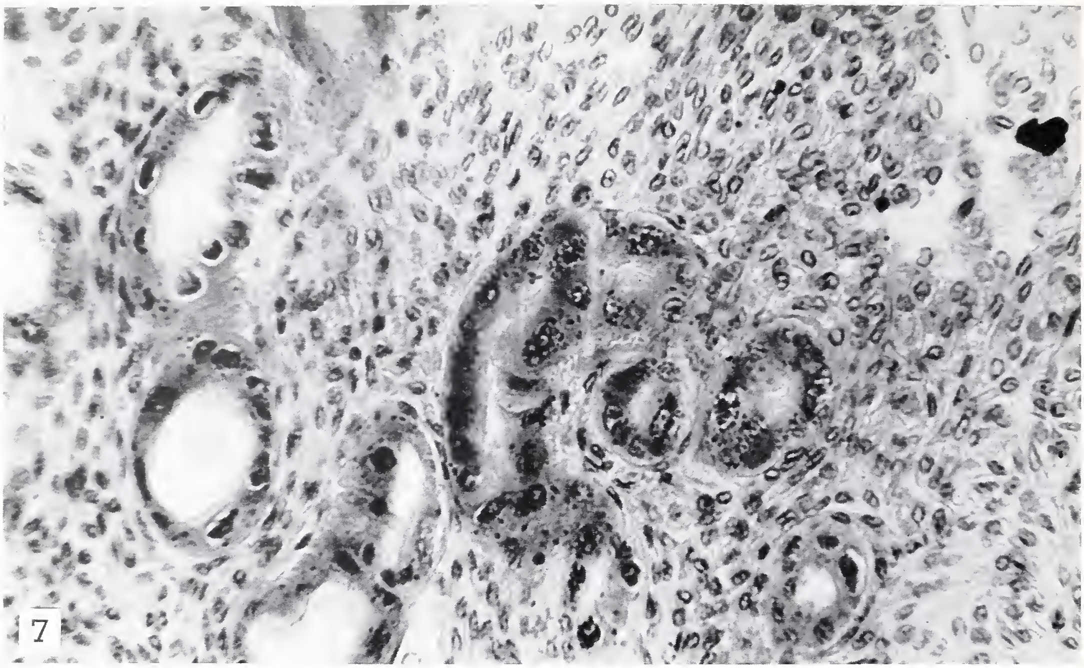


FIGURE 5.—Same as figure 4, with *arrow* indicating longitudinal axis of encapsulated Malpighian tube, from which the new epithelium appears to originate. Hematoxylin and eosin. × 500

FIGURE 6.—Malpighian tubes, cross section, with various cellular changes before encapsulation. MCA diet. Hematoxylin and eosin. × 530



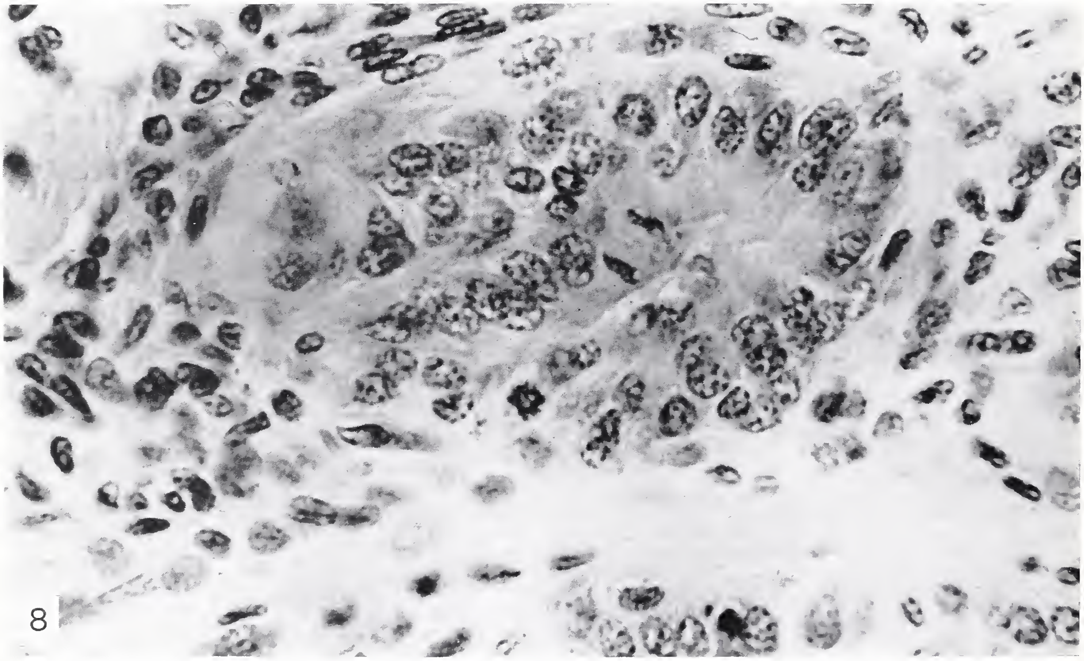
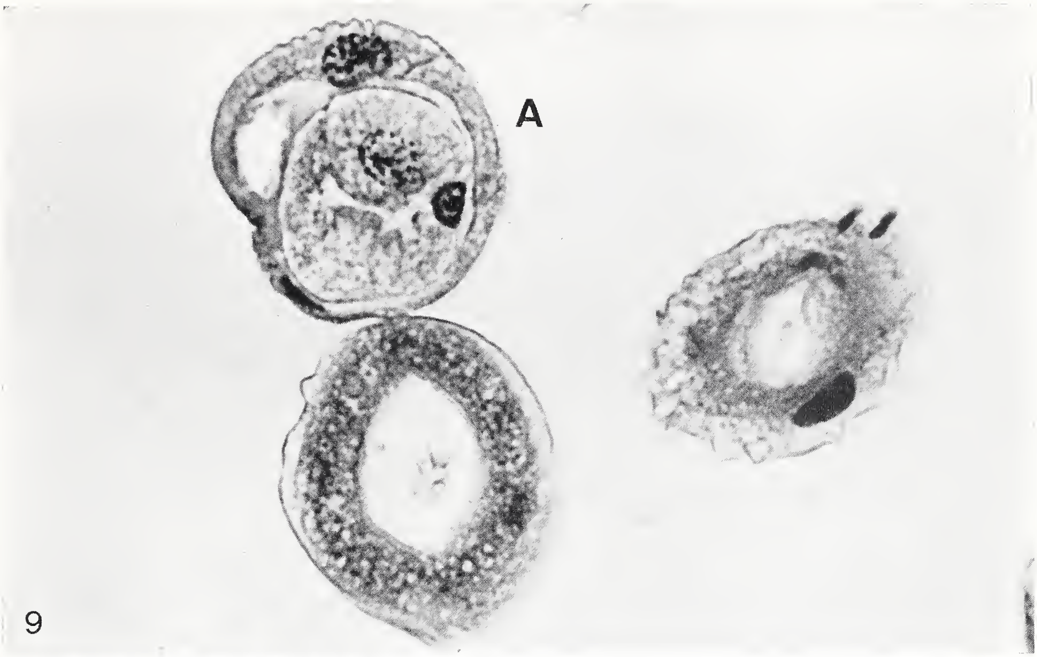


FIGURE 7.—Malpighian tubes, encapsulated by hemocytes. DPAA diet. Hematoxylin and eosin. × 500

FIGURE 8.—Nests of cells proliferating from tube epithelium surviving encapsulation. FL diet. Hematoxylin and eosin. × 800



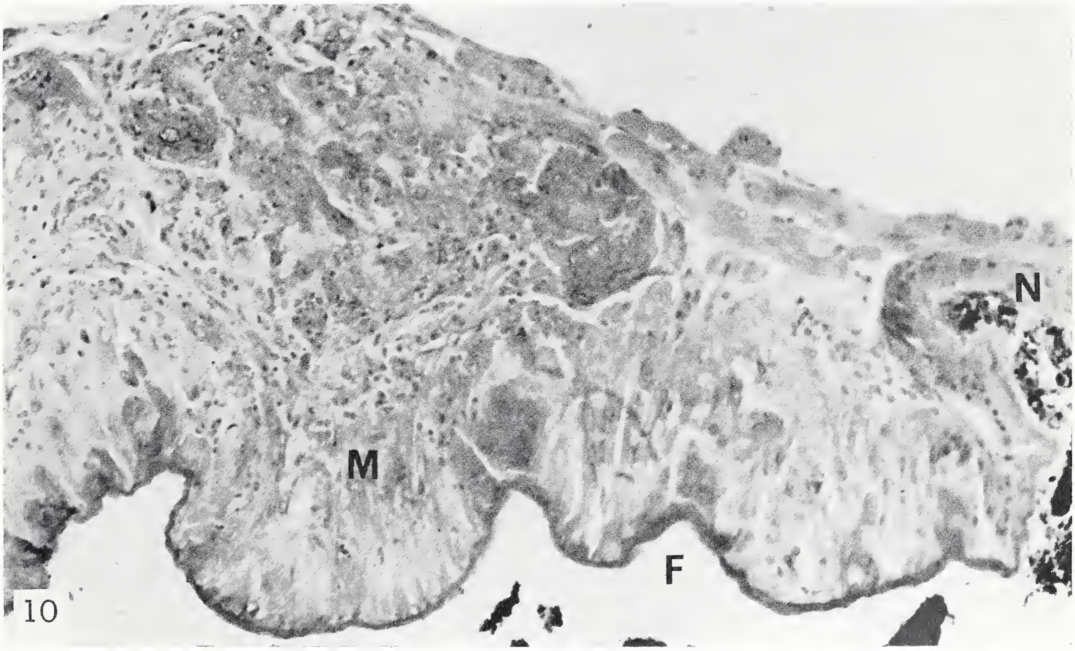


FIGURE 9.—Malpighian tubes, cross section, one of which (A) contains a new tube. MCA diet. Hematoxylin and eosin. × 840

FIGURE 10.—Hindgut wall, longitudinal section, with thick homogeneous layer (M) containing dead cells. Hindgut lumen (F) and normal epithelium (N). DPAA diet. Hematoxylin and eosin. × 190

Abnormal Cellular Responses in *Periplaneta americana* (L.) Resulting From Intracoelomic Injections of Benzo[*a*]pyrene[1]

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Several studies have described cellular responses in insects to chemical carcinogens of vertebrates: *a*) hemocyte encapsulation, with no indication of autonomous growth, of methylcholanthrene and talc crystals inserted into the hemocoel of *Periplaneta americana* (*1*); *b*) increased mutagenic activity in *Drosophila* resulting from exposure to several carcinogenic hydrocarbons (*2*); and *c*) stimulation, under constant chemical exposure, of regenerative gut cells of *Musca domestica* by 2-fluorenamine derivatives (*3*, *4*).

Although many researchers have not hesitated to describe cell responses as tumors (*5*), little progress has been made in insect oncology, largely due to various hemocyte reactions. Difficulties in interpreting possible neoplasms have resulted largely from misleading forms of hemocyte encapsulation. However, the studies of Jones (*6*), Grimstone *et al.* (*7*), and Salt (*8*) form a sound basis for our understanding of the role of hemocytes as a defense mechanism.

This report describes cellular responses, apparently nonhemocytic in origin, initiated by benzo[*a*]pyrene. Developing American cockroach nymphs (*P. americana*), weighing 0.6–0.9 g, were used; thus at least one molt occurred during the arbitrary experimental period. The carcinogen, dissolved in redistilled acetone (2 mg/ml), was introduced as a single intracoelomic injection of 5 μl/insect. Injections were made between the fourth and fifth abdominal sternites with the use of a microinjector holding a 1.0 ml calibrated syringe fitted with a 25-gauge needle. Comparable acetone-injected and untreated individuals constituted the controls. The cockroaches were constantly supplied with pelleted dog food (Ralston Purina Co., St. Louis, Mo.) and distilled water, and maintained at room temperature (23–24°C) under continuous light. Heavy mortality (60–70%), presumably the result of acetone poisoning, was observed in all treated insects and acetone controls 1 or 2 days after injection. Survivors

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[1] Approved for publication by the Director of the Wisconsin Agricultural Experiment Station.

were examined both macroscopically and histologically as early as 2 weeks after injection, and at arbitrary intervals thereafter. The insects were anesthetized with CO_2, and abdominal segments 3–6 were severed with a razor blade and immediately fixed under vacuum in Zenker's solution. Serial cross sections (10 μ) of paraffin-embedded tissues were stained with hematoxylin and counterstained with eosin Y to evaluate treatments and controls.

Histological examination of benzo[*a*]pyrene-treated *P. americana* revealed abnormal cell responses 15, 23, and 28 days after treatment. Such tumefactions occurred in 6 males and 4 females out of 40 roaches examined (25%) (benzo[*a*]pyrene-treated). One response 28 days after treatment displayed mitotic figures (1–2/500 cells) (figs. 1b and 3).

Because of apparently significant differences in the size of the abnormal cells as compared to hemocytes, measurements were made of the areas of 50 abnormal cell nuclei and 50 hemocyte nuclei on the same slide. Only interphase nuclei (abnormal cells) near mitotic figures were measured; thus variation due to "ballooning" and pyknosis was avoided. Hemocyte nuclei that appeared free-floating in the hemocoel, as far from the response as possible, were selected for measurement. The mean value for the abnormal cell nuclei was 58.2 μ^2 compared to 16.8 μ^2 for hemocyte nuclei; $T=19.1$, $P<0.01$ (2-tailed T test). Neither mitosis nor appreciable change in nuclear area has been noted in encapsulation studies (*1*, *8*).

Abnormal cell responses were associated with the midgut (figs. 1a and 1b) and hindgut (figs. 2 and 3). In figure 1b *note* lack of demarcation between the midgut epithelium (E) and abnormal cells (T). Serial sections were studied for a three-dimensional picture of the abnormal responses. False interpretation from artifacts due to oblique sectioning angles was thus ruled out. Abnormal cells also were associated with hindgut circular muscle cells and cuticle epithelium (figs. 2 and 3). In addition, a brown melanin-like material (fig. 1a) was associated with both treated roaches and controls. A preliminary qualitative test for chitin (KOH and iodine) was negative. Acetone-treated insects, 2 weeks after treatment, showed brown amorphous material typically encapsulated by hemocytes. Early examination (4–8 days after treatment) revealed encapsulation of debris in both treated cockroaches and controls. We believe that the brown material represents the site of the local tissue damage resulting from the acetone vehicle, because such a reaction was always found near the site of injection.

Because of the abnormal frequency of mitotic figures and apparent nonhemocytic nature of these cells, investigations are being conducted to elucidate possible neoplastic aspects of these tumefactions.

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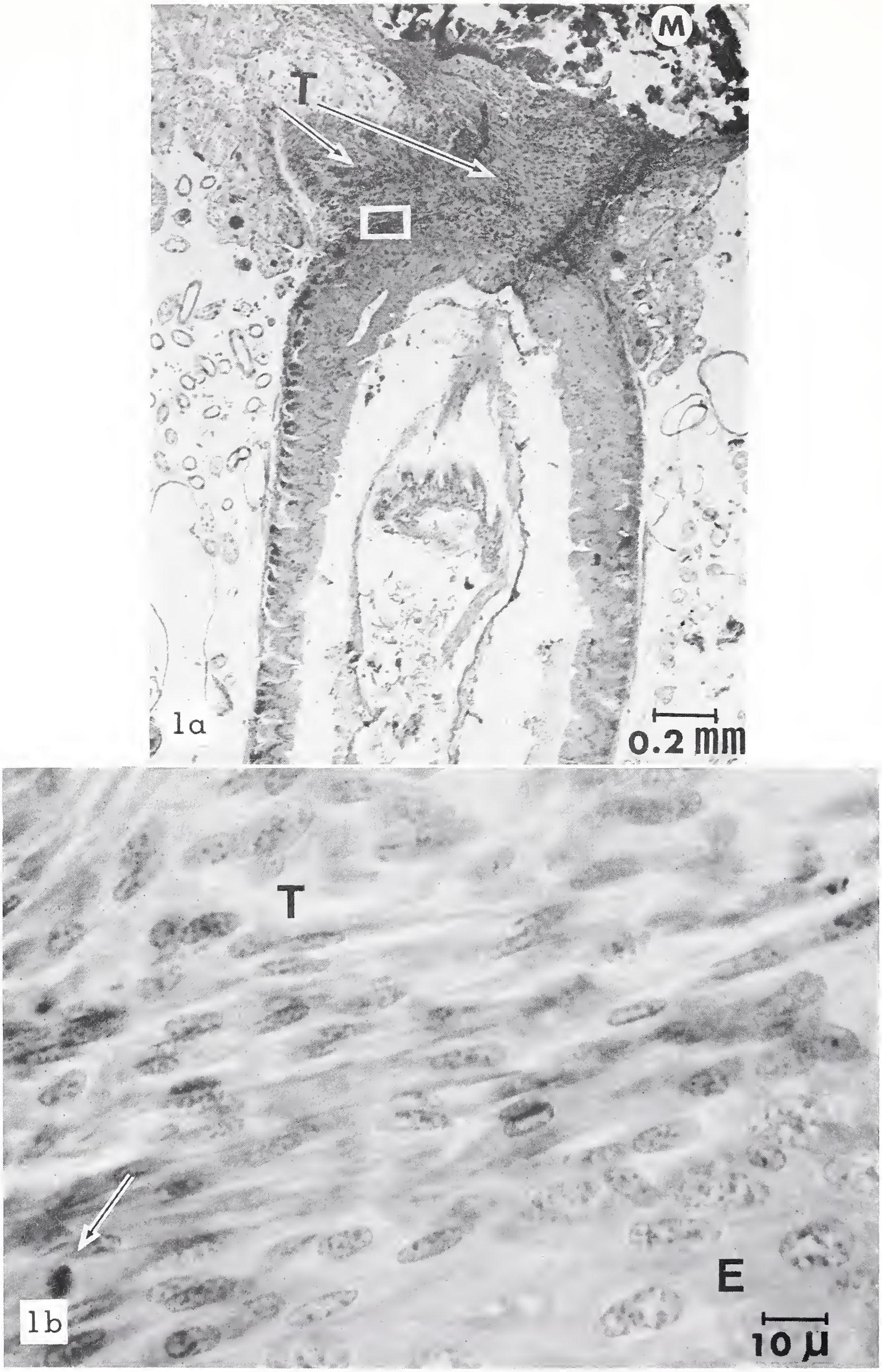

FIGURE 1.—*a*) Tumefaction (T) associated with midgut (♀ *P. americana*) 23 days after treatment with benzo[*a*]pyrene. *Note* melanin-like material (M) associated with abnormal response. *b*) Enlargement of area in *rectangle* in figure 1a. *Note* lack of demarcation between midgut epithelium (E) and abnormal cells (T), and one cell in metaphase (*arrow*). Hematoxylin and eosin.

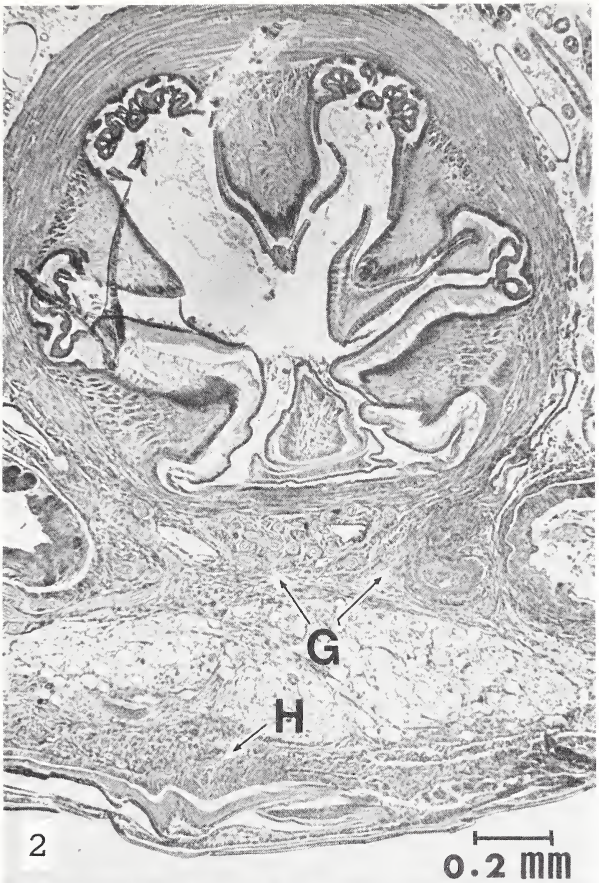

Figure 2.—Abnormal cell response (G) associated with hindgut and hydrodermis (H) in developing ♂ 15 days after treatment. Hematoxylin and eosin.

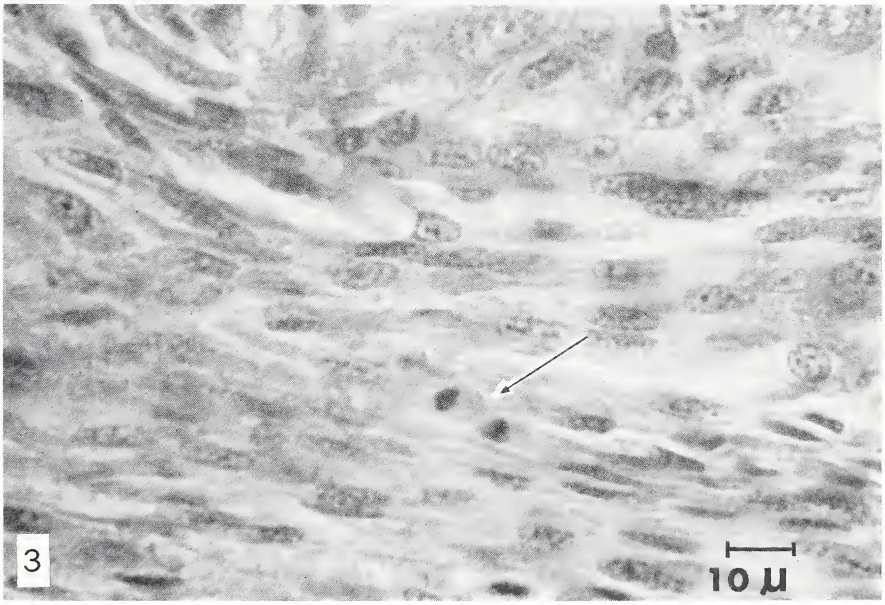

Figure 3.—Abnormal cells associated with hindgut (♂) 28 days after treatment. *Note* cell in early telophase (*arrow*). Hematoxylin and eosin.

Rectal Tumefactions in the Cockroach, *Periplaneta americana* (L.)[1,2]

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ONE OF the most common sites of abnormal growths in adults of our laboratory strain of *Periplaneta americana* (L.) has been the rectal pads. This has also been observed for *Blattella germanica* (L.) (*1*). An examination of adult *P. americana* of various ages revealed that incidence of these growths increased with age, and at 9–10 months possibly was higher in males than in females, especially on the basis of the number of rectal pads affected. Although mortality data were unavailable, such differences between sexes may be due to the development rates of the growths.

In studies to date, at least two types of growths have been observed (fig. 1). Type I is a small compact area, slightly whiter than normal surrounding tissue. A compact, round, dark-brown mass within this growth is generally evident. Type II is very white and opaque, irregular in shape, and may involve the entire rectal pad. In late stages of development, diffuse amber patches appear on the inner surface of the pad. Both types may occur in the same rectal pad.

Type I begins to develop when hemocytes encapsulate unknown material between the rectal muscularis and the rectal epithelium (fig. 2). Initially this does not involve the latter tissue, but with continued hemocyte encapsulation and necrosis of inner hemocytes, the regenerative cells of the epithelium are involved and add to the necrotic mass (fig. 3). The mass and additional encapsulating hemocytes are moved toward the rectal lumen, thereby involving the epithelial cells closest to the lumen (fig. 4). From the unaffected regenerative epithelial cells at the periphery, a new epithelium proliferates under the mass, which in turn is sloughed into the lumen.

Although the developmental stages of type II are not completely known, the white opaque material on the inner rectal pad surface appears to be a product of the rectal epithelium and is similar to that observed in hindgut disorders. Hemocytes invade the area, and these and the epithelium are destroyed. A new epithelium is formed as in type I (figs. 5 and 6). Processes involved in both types appear to be examples of an insect defense mechanism for the displacement into the rectal lumen of disorders in and near the rectum and subsequent repair of the epithelium.

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[1] This investigation was supported by National Science Foundation grant GB-7446.

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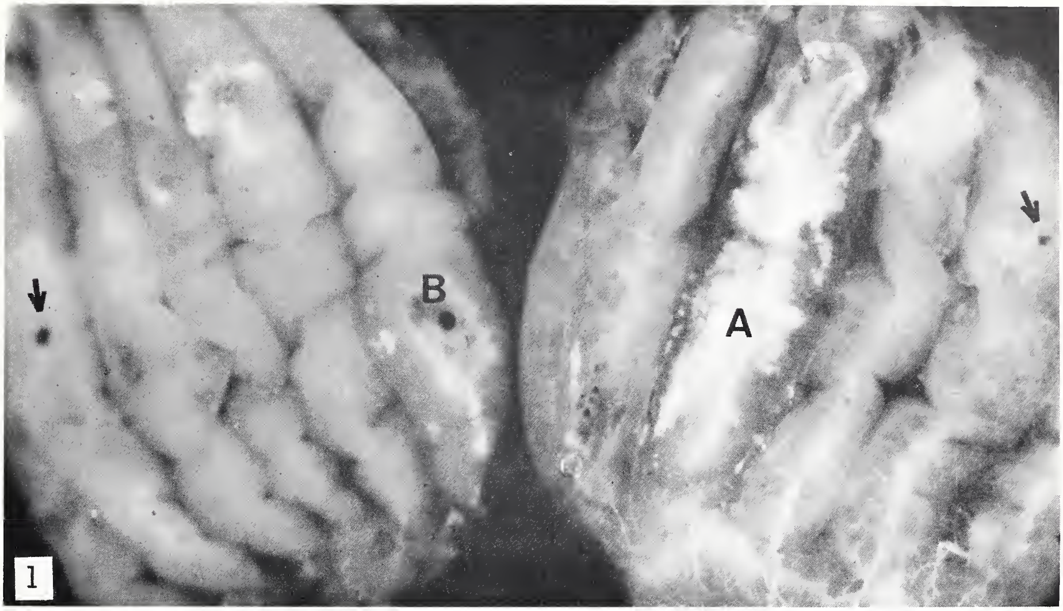

FIGURE 1.—Internal view of 2 opened recta, with type I growths (*arrows*), type II growth (A), and possible mixture of types (B). Tissue unfixed. × 25

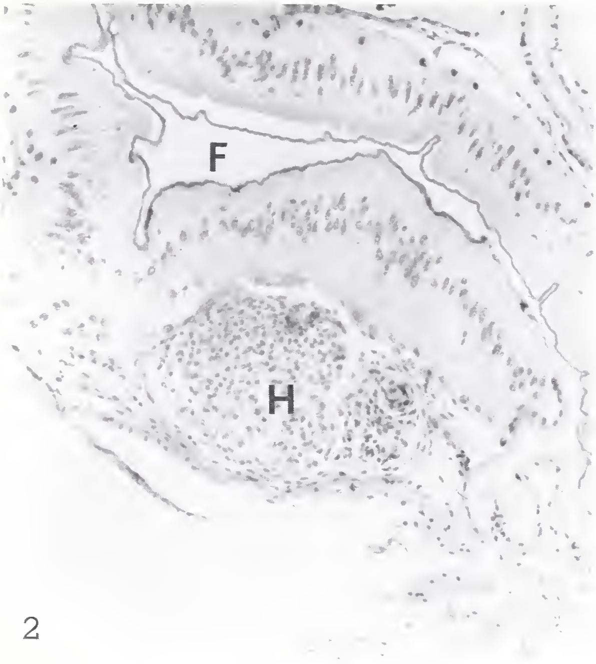

FIGURE 2.—Rectal pad, longitudinal section, with type I growth. Hemocyte mass (H) between muscularis and epithelium; rectal lumen (F). Hematoxylin and eosin. × 130

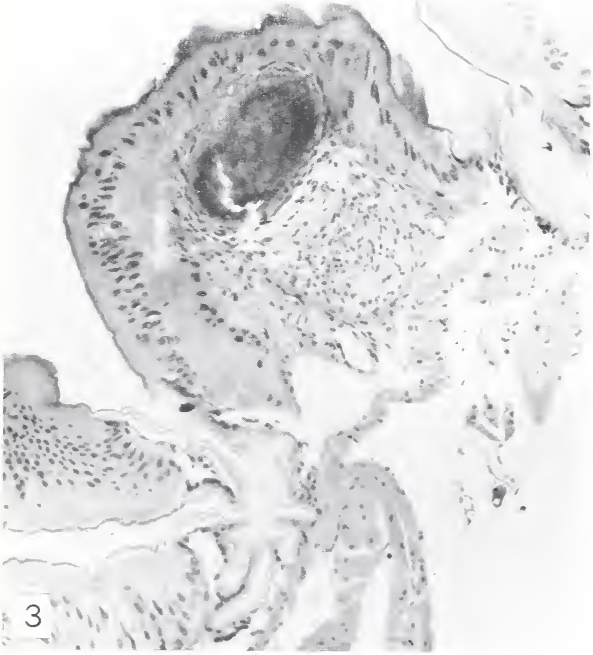

FIGURE 3.—Rectal pad, cross section, type I growth with necrotic mass of hemocytes involving regenerative cells of epithelium. Hematoxylin and eosin. × 130

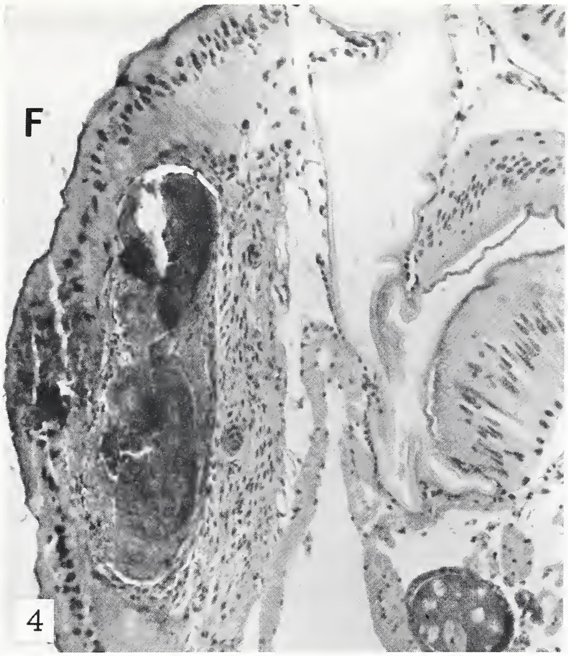

Figure 4.—Later stage than shown in figure 3, longitudinal section with mass involving remaining epithelial cells above; lumen (F). Hematoxylin and eosin. × 130

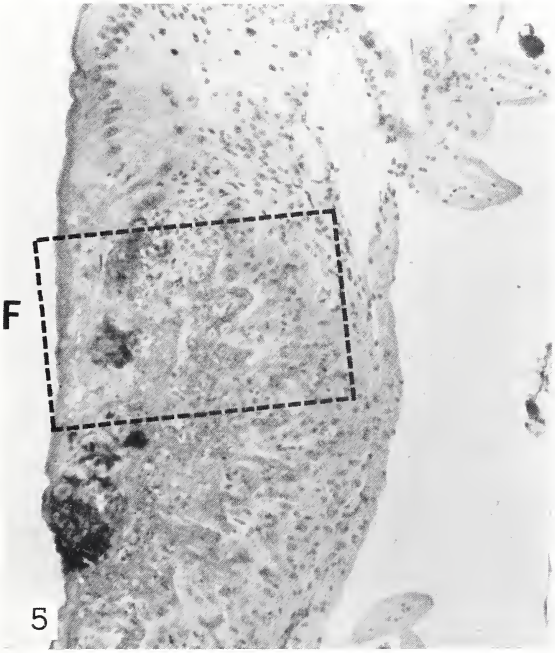

Figure 5.—Rectal pad, cross section, late stage mixed types I and II growth, with development of new epithelium; lumen (F). Hematoxylin and eosin. × 130

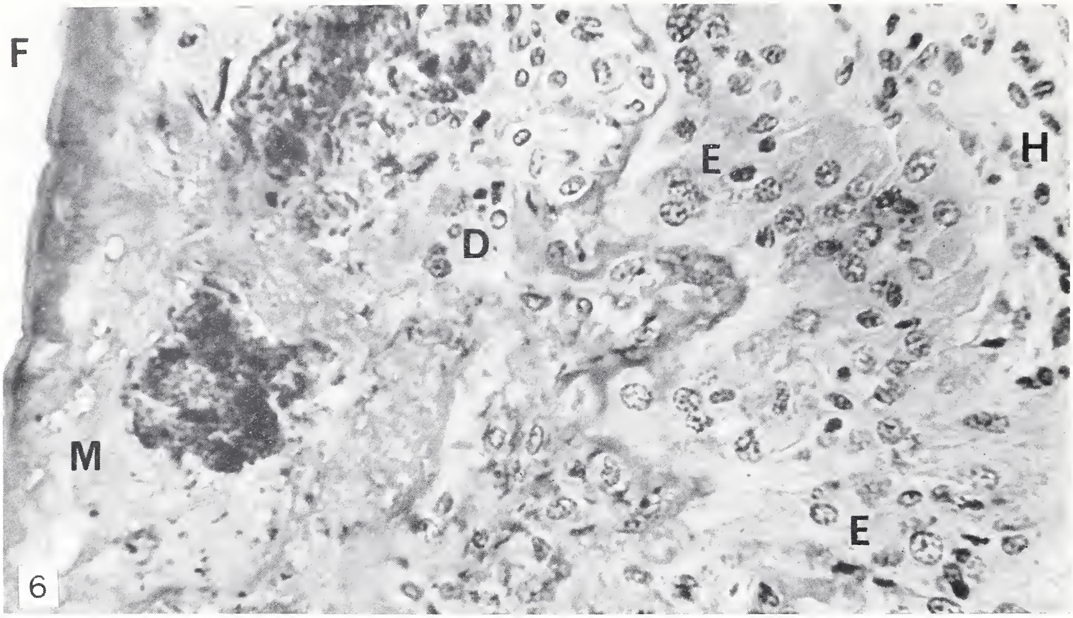

FIGURE 6.—Area outlined in figure 5. New epithelium (E) formed below hemocytes (D) which previously had invaded affected epithelium with opaque material (M) next to lumen (F). Additional layer of hemocytes (H) which may ultimately reenter circulation. Hematoxylin and eosin. × 460

Possible Ovarian Influence on Tumor Development After Nerve Section in *Periplaneta americana* (L.)[1]

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SUMMARY

In the cockroach *Periplaneta americana*, section of the recurrent nerve induces tumor development in the alimentary canal. Presence of functional ovaries in the female results in more rapid and extensive tumor growth on nerve section. This suggests a relationship between functional ovaries and tumor development in the cockroach.—Nat Cancer Inst Monogr 31: 459–463, 1969.

INFORMATION ON true tumors in insects is scanty. In general, insects react to injuries or irritation by accumulating cells, especially hemocytes, which produce tumor-like formations at the site of injury. Defining a tumor is difficult even in vertebrates, despite the wealth of available literature. Increased volume and irregularities of nuclei, multiple nucleoli, anomalies of mitoses, and cytoplasmic basophilia show up in most, though not in all, tumors. To a histologist studying insect material, including tumors, such characteristics appear in a wide variety of situations, which makes it inadvisable for him to generalize.

Neoplasms in insects were recently reviewed by Harshbarger and Taylor (*1*). In the category of induced tumors, Scharrer (*2–4*) clearly delineated tumor induction in the alimentary canal of the cockroach *Leucophaea maderae* by severing the recurrent nerve. Her studies revealed that about 75% of sectioned insects, both nymphal and imaginal, developed tumors and that the incidence of tumors did not differ significantly in the two sexes. The mortality rate of the tumor-bearing cockroaches showed significant sex differences—males as a group surviving longer than females, a difference that disappears in castrated insects.

In the cockroach *Periplaneta americana*, severing the recurrent nerve induced tumor growth in the alimentary canal (*5*). The tumors affected the salivary apparatus, gastric ceca, and midgut. Histologically, the different tumors had a characteristic pattern of structure. The cells were

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[1] Part of the work was done with financial assistance from the Ford Foundation.

somewhat distended and the nuclei augmented in size. As growth proceeded, the nuclei became hyperstainable. Hyperstainability was commensurate with a shrinkage of nuclei; nuclei were elongated, chromatin was clumped, and perinuclear spaces developed. Nuclear fragmentation and mitoses were in the swollen mass. The epithelial lining gave rise to tumor formation. The nidi became involved in the growth of tumor in the midgut (fig. 1).

A well-defined relationship exists between the sex and the rapidity and involvement of tumor growth. Section of the recurrent nerve in a normal female resulted in the growth of well-defined tumor within 28–30 days, but there was a natural delay in the development of tumor in a male, the tumor only becoming distinct 45 days after the operation. In the female, the reproductive cycle continued uninterruptedly, new oothecae being produced once in every 3–6 days. Also, the rate of survival was poor in males, and of the surviving insects only about 75% developed tumors. In females the survival rate was 100%, and 95% of them developed tumors.

In females in which the ovaries were removed, nerve section resulted in an extremely slow rate of development of tumor, from about 105–110 days after operation. This was also true for insects in which the recurrent nerve had been severed within 2 or 3 days after ovariectomy. But if the nerve had been severed at least 1 week after ovariectomy, no tumors developed, even after 210 days. Of the scores of insects studied, only one examined after 92 days developed a tumor, but an ovarian follicle had been accidentally left within this insect.

In males, in which ovary or follicle had been transplanted, the tumor grew with a marked rapidity and was well defined and fairly extensive within 24 days.

In an insect with an inactive ovary, like the female nymph or isolated, newly emerged female adult, nerve section did not induce tumor growth. However, in the last instar nymph in certain instances, some tumor growth occurred. A brief summary of the experiments done and the results obtained is given in table 1.

The recurrent nerve is a pathway for neurosecretory material in insects. In *P. americana*, it is not conspicuous, because occasionally extremely fine granules are present, which on analogy alone could be described as neurosecretory material (fig. 2). However, in ovariectomized insects, severance leads to an accumulation of neurosecretory material at the stump of severed nerve, which becomes progressively conspicuous only after 100 days. Generally, slender nerves originate as regenerates from the cut end, which run posteriorly or posterolaterally. These fibers also show granules and colloids suggestive of neurosecretory material (fig. 3). In insects growing the tumor as a result of nerve severance, the median neurosecretory cells and the tracts pile up neurosecretory material; in males, where such an occurrence is extremely rare, this phenomenon becomes marked when they receive ovarian transplants.

These findings suggest a relationship between the functional ovary and tumor development in *Periplaneta* when the nerve is severed. The ovary

TABLE 1.—Summary of experiments: *Periplaneta americana*

Sex and experiment	Tumor	Neurosecretory system	Remarks
Females			
RN* cut	++	—	
RN cut, ovary removed	—	+	
Newly emerged, RN cut	—	Faint	
Males			
Nymph RN cut	—	—	
Nymph with transplant of ovary, moults, RN cut later	++	+	
Adult: RN cut	+	—	Slow development of tumor
RN cut +ovary implant	++	+	Rapid growth of tumor

*RN = recurrent nerve.

affects the neuroendocrine system of the animal, and the influence of the corpora allata and the ovaries is well known. The hormone elaborated by the corpora allata is produced in both sexes, though to an increased extent in females exercising the gonadotropic function. There is a slight increase in size of the corpora allata in male insects receiving ovarian transplants. But allatectomy results in growth of tumor in females when the recurrent nerve is cut.

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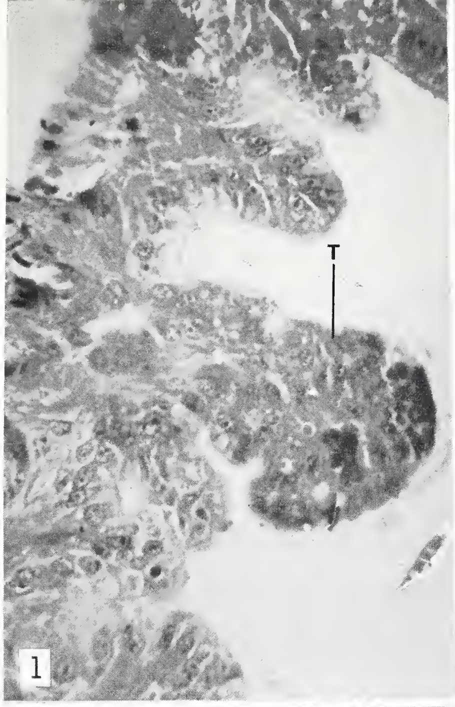

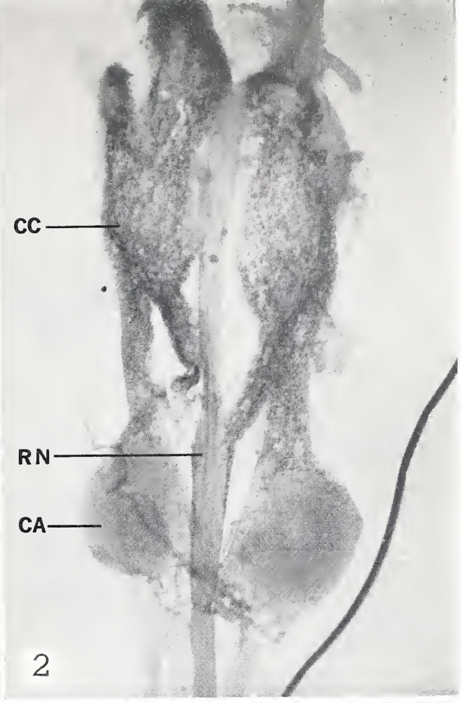

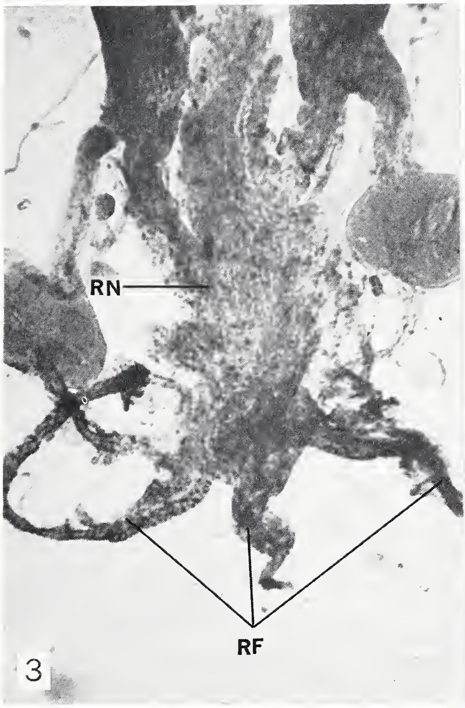

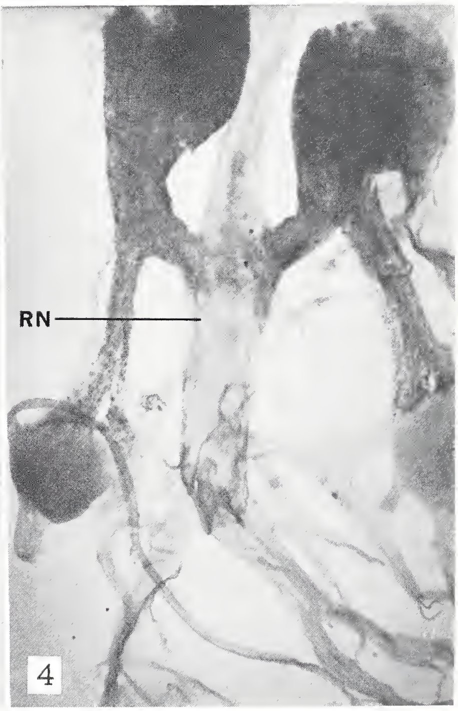

Abbreviations: **CA: corpus allatum; CC: corpus cardiacum; RF: regenerates of nerve; RN: recurrent nerve; T: villus turning tumorous.**

FIGURE 1.—Villus of epithelial lining of the midgut of adult female *Periplaneta* 58 days after nerve severance, showing beginning of tumorigenesis. Heidenhain's iron hematoxylin. × 375 FIGURE 2.—Scanty distribution of neurosecretory material in the recurrent nerve of control adult female. Wholemount: performic acid-victoria blue. × 80 FIGURE 3.—Extreme accumulation of neurosecretory material in the nerve of ovariectomized adult with recurrent nerve cut. Three regenerating fibers are loaded with material. Wholemount: performic acid-victoria blue. × 100 FIGURE 4.—No accumulation of material in female with ovaries but with recurrent nerve cut. Same age as female in figure 3. Wholemount: performic acid-victoria blue. × 85

Histology and Transmission of *Locusta migratoria* (L.) Tumors (Insecta, Orthoptera)

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SUMMARY

The tumors appearing in *Locusta migratoria* L. originate from abnormal proliferation of the epithelial cells. This is particularly evident in the embryonic cells of regenerative nidi in the midgut wall. These tumors can be transmitted by injections of hemolymph from tumorous animals, of crushed tumor homogenates, or of either cell-free extracts or of ribonucleic acid extracted from tumorous organs. Heating of tumor filtrates or of nucleic acid preparations as well as using ribonuclease resulted in the loss of this infectious capacity. Using hemolymph or filtrates of tissues of healthy insects or filtrates of hemocytic aggregates (aggregates around cellophane implanted in the hemolymphatic cavity) did not result in tumor formation.—Nat Cancer Inst Monogr 31: 465–473, 1969.

SEVERAL INVERTEBRATES have shown spontaneous lesions referred to as "tumors" or "cancer," but most of them are agglutinated hemocytes or, in other words, granulomas, nodules, or cysts.

Following Pflugfelder's (*1–6*) and Scharrer's (*7–12*) original work and the publication of reviews (*13–20*), only a few experimental studies have been conducted on orthopterans until quite recently. Harker (*21*) obtained tumors in the midgut of the American cockroach exposed to continuous subesophageal hormone by properly timed implantations of active subesophageal ganglia. However, according to Nishiitsutsuji-Uwo and Pittendrigh (*22*), who were unable to corroborate her work, these tumors could not have a neuroendocrine basis. Two other experiments gave positive results in producing tumors in cockroaches: the application to wounds of farnesyl methyl ether, which sometimes causes treated animals to develop genital tumors (*23*), and the ligation of salivary ducts reported by Sutherland (*24, 25*). Edwards *et al.* (*26*) repeated Sutherland's work and obtained similar results: Cysts arose from hemocytes encapsulating the degenerating cells, but neither mitotic figures nor DNA synthesis was detected. I reported a hypothesis for interpretation of Sutherland's work in 1967 (*27*). In a lepidopteran, Hukuhara (*28*) reported that injection of Tipula iridescent virus into *Bombyx mori* caterpillars incited an abnormal proliferation of hypodermal cells of the integument. Finally, other results are due to Day (*29*), L'Helias (*30–32*), Hema (*33*), and Johansson and Schreiner (*34*).

This paper summarizes the most important results of my studies of *Locusta migratoria* tumors which were given in considerable detail in 1967 (*27*) and includes results from the study of the transmission of tumors by a nucleic acid extract from tumors (*35, 36*).

EXPERIMENTAL RESULTS

Among *L. migratoria*, nerve severance induced development of tumors in the digestive tract (*37, 38*). The tumors appeared in 7% of the animals (54 of 770) after a lag time of 20–30 days (for a lifetime of 70 days) following severance of one of the sympathetic or gastric nerves. The average number of tumors was 2.6 for each injured animal, but incidence was increased by bilateral or unilateral section at an early nymphal stage. Severance of the splanchnogenital nerve provoked the development of tumors in the hindgut and the genital ducts, whereas tumors never appeared after severance of the motor nerve.

But in another strain of *L. migratoria*, obtained from the Anti-Locust Research Center of London, the tumor incidence was different: Only 1 of 323 insects had tumors after unilateral severance of the nerve, and 10 of 74 after bilateral severance.

Histology

Tumors were located in the digestive tract where they developed by abnormal multiplication of the epithelial cells. Mitosis was noted, particularly in the embryonic cells of regenerative nidi in the midgut wall and in the gastric ceca. These cells divided actively, but instead of forming an epithelium they remained undifferentiated and kept multiplying (figs. 2 and 3). In the ectodermal organs, the epithelial cells were also the ones that multiplied (*27, 39*).

Thus the early development of tumors is characterized by an intense epithelial proliferation which stimulates the defensive mechanism of the insect to encapsulate the lesion with hemocytes.

Comparative Study of Tumors and Inflammatory Processes

Several inflammatory processes were studied: phagocytosis and formation of hemocyte nodules after injection of India ink (*40*), encapsulation of implanted cellophane by hemocytes (*40, 41*) (fig. 1), wound healing in the digestive tract after mechanical injury (incision) (*40*), and poisoning by sodium arsenite, toluene, and the vertebrate carcinogen methylcholanthrene (*40*).

Hemocyte encapsulation reactions are characterized by a lack of cellular proliferation, arising only by agglomeration of hemocytes. Hemocytes never incited cell proliferation in the encapsulated organs, which indicates that hemocyte encapsulation is entirely a defensive mechanism. On the contrary, tumors developed; the epithelial cells became invasive, metastasized, and killed the insect (*42*).

Transplantation

When tumors were transplanted, 60% of the host animals developed metastases in the foregut, midgut, ceca, salivary organs, and genital ducts.

Pieces of healthy tissue from the digestive tract were implanted in the hemocoel, and the following results were obtained: 1) The tissue degenerated and was encapsulated by hemocytes (in this case, other hemocyte nodules appearing in the abdomen, or any other organ, such as the malpighian tubules, degenerated); 2) it survived without alteration; 3) it became an innervated organ—the epithelial and regenerative cells of the endodermic nidi multiplied and it was encapsulated by hemocytes. If this is true, implants transform into tumors in the same way as after other procedures. Tumors were present in the other organs of the host in 195 of 1,456 animals, or 13.4%. A minimum lag time of 10 days was necessary before metastasis occurred: This is a fundamentally distinctive difference between tumorigenesis and infection. Metastases averaged 1.8 per animal (344 tumors for 195 injured insects). The gastric cecum had the highest tumor incidence, followed by that of the midgut, the hindgut, the foregut, and genital ducts. According to Sutherland (*25*), these formations may have resulted from injury at operation or hemolymph clotting. I believe these formations resulted from metastasis; figure 4 shows infiltration of tumor cells into healthy organs.

Hemolymph from tumorous insects is infectious when injected into healthy insects (*43*). Physiological salt solution or filtered hemolymph is inactive. Though heat causes the infectious capacity to disappear, the hemolymph of tumorous insects can be infectious after one or more passages in insects remaining apparently healthy.

Crude tumor extracts transmit the tumors, as well as cell-free extracts after filtration [pore diameter$=0.45$ and $0.1\ \mu$ (*27*, *43*)]. Infection was obtained by two classic methods: injection of the infectious material into the hemolymphatic cavity, and by oral administration (table 1).

Tumors can be transmitted by a pathogenic element localized in the tumor itself or in the hemolymph of injured insects, but transmission is impossible by a filtrate of tissues and the hemolymph of healthy insects, a tumor filtrate (pore diameter$=0.05\ \mu$), and a filtrate of hemocytes encapsulating implanted cellophane. The element responsible for tumor transmission is destroyed by heat but it is not affected by preservation in the cold.

Tumors can be transmitted by a nucleic acid extracted from the tumors but absent in the healthy animals. The experiment was performed on over 1,700 animals (*36*). The effects of the injection of various nucleic acid preparations are given in table 2.

Nucleic acids extracted from apparently healthy insects (which have had a gastric nerve cut but have not developed any tumors) appeared to be infectious. We have already commented on this (*36*) and suppose that severance of the sympathetic nerve induced the development of infectious material. Certain insects remaining apparently healthy might have this material, which could cause the development of tumors in other animals that received injections.

The action of nucleases suggests that the material responsible for the transmission of tumors is a ribonucleic acid (table 3).

TABLE 1.—Effects of the injection of various materials into *Locusta migratoria*

Material injected	Number of insects in experiment	Number of tumorous insects	Number of tumors
Tumor homogenate	61	7	14
Tumor filtrate (pore diameter = 0.45 μ)	658	123	583
Tumor filtrate (pore diameter = 0.1 μ)	118	6	17
Tumor filtrate (pore diameter = 0.05 μ)	44	0	0
Tumor filtrate (pore diameter = 0.45 μ) after heating	35	0	0
Filtrate of muscle of healthy animals (pore diameter = 0.45 μ)	54	0	0
Filtrate of fat body of healthy or tumorous insects (pore diameter = 0.45 μ)	102	0	0
Filtrate of hemocytes encapsulating cellophane (pore diameter = 0.45 μ)	101	0	0
Ringer's physiological solution	30	0	0
Hemolymph of healthy insects	98	0	0
Hemolymph of tumorous insects	997	142	701
Hemolymph of tumorous insects after heating (70°C for 15 min.)	49	0	0
Hemolymph of tumorous insects after filtration (pore diameter = 0.45 μ)	40	0	0

TABLE 2.—Effects of injection of various nucleic acid preparations into *Locusta migratoria*

Nucleic acid extracted	Number of insects in experiment	Tumorous insects	
		Number	Percent
From digestive tubes of insects showing tumors 20–30 days after section of a gastric nerve	472	94	19. 9
From digestive tubes of insects apparently healthy after section of a gastric nerve	242	66	27. 3
From digestive tubes of insects showing tumors 12 days after injection of tumorous nucleic acids extracted from the 94 tumorous insects of the first series	436	101	23. 1
From digestive tubes of healthy insects	108	2	1. 8
From muscle of healthy insects	49	2	4. 1

TABLE 3.—Action of nucleases on the infectious nucleic acid

Material injected	Number of insects in experiment	Tumorous insects	
		Number	Percent
Filtered homogenate (pore diameter = 0.45 μ)	30	7	23. 3
Nucleic acid extracted from this homogenate	160	38	23. 7
Nucleic acid treated with ribonuclease	44	1	2. 3
Nucleic acid treated with deoxyribonuclease	68	16	23. 5

CONCLUSION

Because so few tumors are observed in invertebrates, clearly this subject remains confused.

Spontaneous "tumors" as well as most melanotic pseudotumors in *Drosophila* are hemocytic encapsulations of parasites or degenerate tissue and can be called nodules, cysts, or granulomas. They can be reproduced by injection of inert powders, implantation of cellophane, etc.

These inflammatory formations mobilize only the hemocytes and are characterized by lack of cellular proliferation, metastasis, and transmission by a filtrate. The tumors of *L. migratoria* are epithelial and result from proliferation of the epithelial cells of an injured organ. They can be transmitted by cell-free extracts. It remains difficult to homologize insect tumors with cancer of vertebrates. For descriptions of different pathologic conditions that appear in insects to have real meaning, such descriptions must take into account mitotic divisions, the importance of hemocyte immigration, the possibility of transmission, invasiveness of growth, etc. Only when this is done can pathologic comparisons be made.

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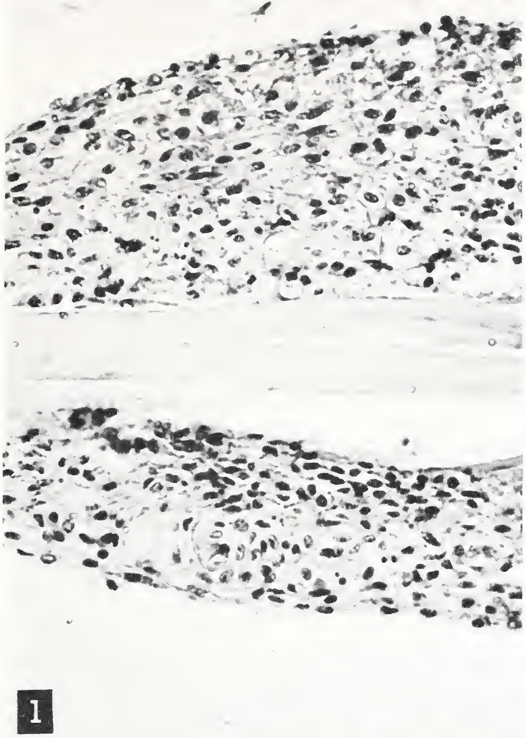

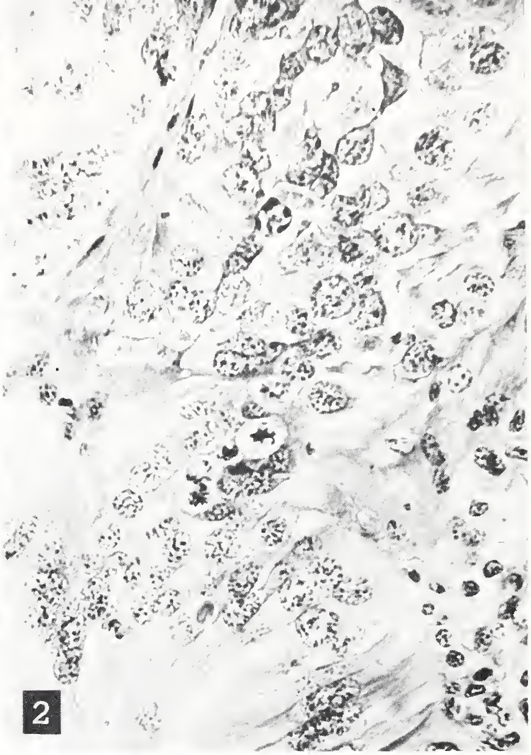

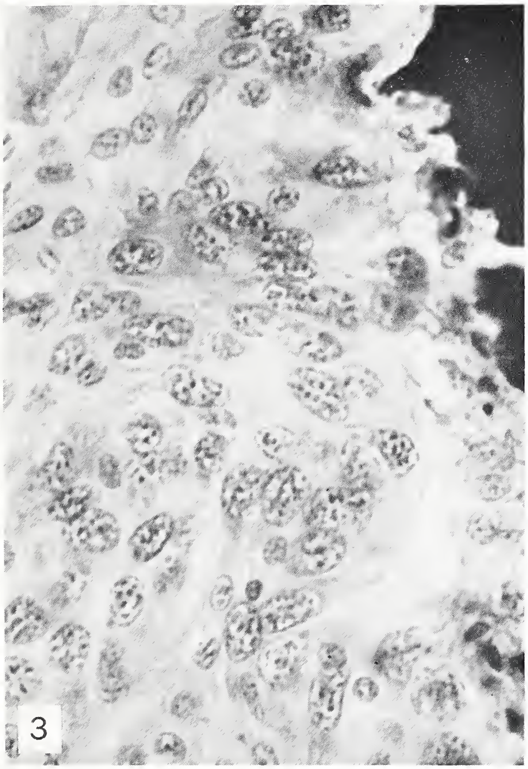

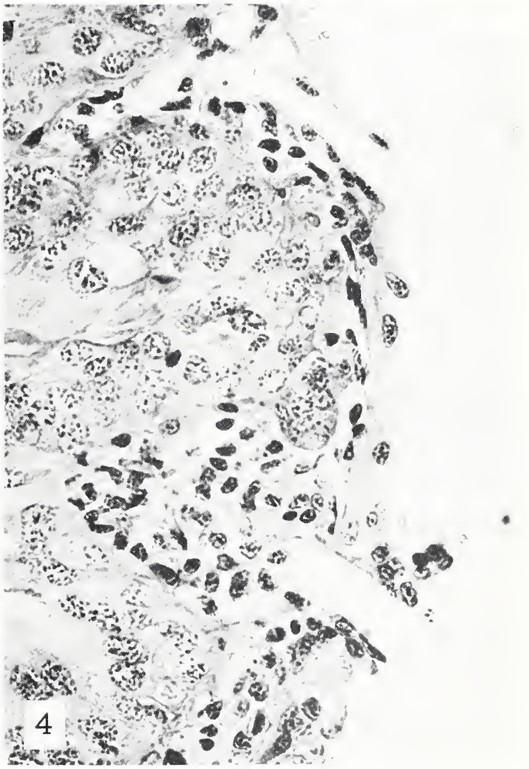

FIGURE 1.—Hemocyte encapsulation of cellophane 4 days after implantation. FIGURE 2.—Cellular proliferation of midgut epithelium after nerve severance. Hemocytes arrive at *right* (*below*). Normal midgut epithelium has only one layer of cells. FIGURE 3.—Oviduct. Normal epithelium has one cellular layer. Here, after epithelial proliferation, the epithelium shows several layers of cells. *Note* irregular shape of the nucleus. FIGURE 4.—Invasive tumor cells in healty cecum. These cells infiltrate along a trachea.

Formation of Cysts as a Defensive Reaction in Pupae and Adults of *Neodiprion swainei* Midd. (Hymenoptera: Tenthredinidae)

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Because of the nature of their formation and structures, the bodies that we first observed in 1964 in the intestinal cavity of prepupae and in the abdominal cavity of adults of *Neodiprion swainei* Midd. were referred to as cysts (*1*, *2*). Even though Bird (*3*) referred to similar bodies found in *Diprion hercyniae* as tumors and associated them with the polyhedral virus of this insect, it was observed that the *swainei* viral infection did not induce the process of cyst formation, but that cysts occurred in direct proportion to the number of individuals supporting viral and bacterial infections or other injuries. For instance, 10% of the adults from a virus-free population contained cysts, among which none contained polyhedra, 6% contained bacteria, and 1% spores of fungi, and 3% were negative. Eighteen percent of the adults from a population sprayed with virus contained cysts, among which 10% contained polyhedra, 2% bacteria, and 0.5% spores of fungi, and 6% were negative. Neilson (*4*) uses the term "tumor-like bodies" for the bodies found in *D. hercyniae* but does not share Bird's opinion (*3*) that viral infection is responsible for the formation of cysts.

The cysts were dark brown-to-black and of various shapes. They measured from 0.8 × 0.8 × 1.0 mm to 1.0 × 1.0 × 2.5 mm (fig. 1). Histologic studies of cysts revealed that no cell proliferation or giant swollen cells and no necrotic pigmented center were present. The cysts looked like a purse made of a membrane, 50–80 μ thick, composed of many layers of denucleated, flattened, spindle-shaped cells which surrounded compact masses of well-preserved structures of the cells of the midgut epithelial tissues, or tissues of the midgut epithelium that had been lysed to various degrees, or tissues that had been completely lysed and formed masses of amorphous, soft, jelly-like matter. The multilayered membrane probably resulted from the precipitation of hemocyte-like cells (figs. 2, 3, and 5). However, it is also possible that this membrane originated partly from an accumulation of the material destined for the formation of the gut peritrophic membrane. Since the virus of this insect developed only in the nuclei of the cells of the midgut epithelial tissues of the larvae, it was possible to observe pockets of polyhedra in the masses of amorphous tissues in the cysts (fig. 4).

Various analyses were performed on the cysts during the spring of 1968 to determine their concentration of uric acid, glycogen, and calcium oxalate. Preliminary results revealed that the concentration of uric acid in the cysts was 10 times higher (7.5 mg/g) than that in the insects, which varied between 0.2 mg and 0.5 mg/g. The concentration of glycogen and of calcium oxalate in the cysts was similar to that in the host.

From these results we drew the following conclusions on the formation and nature of the cysts. When healthy larvae transform into eonymphs and later into adults, the midgut epithelial tissues remaining in the intestinal cavity undergo histolysis and phagocytosis through action of a complex of histolytic enzymes. This is a normal defensive process which liberates the elements that have ceased to be integral parts of the organism. In contrast, when the host is affected by exogenous or endogenous factors, such as physiological injuries or infectious diseases, the midgut epithelium cannot undergo complete histolysis once it is liberated in the intestinal cavity, probably because of either a disturbance in the normal balance of enzymes causing histolysis or interference between these enzymes. Such unlysed tissues of the midgut become extraneous matter in the intestinal cavity and induce another defense mechanism, very similar to the phenomenon of parasite egg encapsulation (fig. 6).

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PLATES

FIGURE 1.—Cysts recovered from pupae and adults.

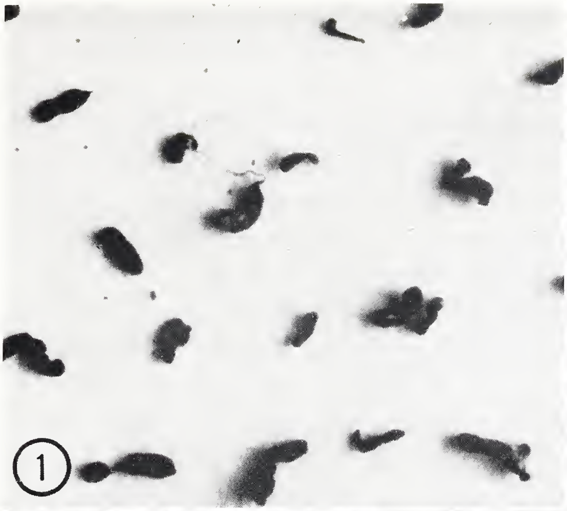
FIGURE 2.—Longitudinal section of a cyst showing the multilayered membrane covering the mass of amorphous jelly-like matter. × 250

FIGURE 3.—Multilayered membrane. × 500

FIGURE 4.—Pocket of polyhedra in cyst. P = polyhedra. × 1500

FIGURE 5.—Degenerate, spindle-shaped, hemocyte-like cells showing the fibrous, chitinous appearance of the cytoplasm.

FIGURE 6.—Transverse section of an encapsulated egg of *Neodiprion swainei* parasite showing the same multilayered membrane as observed with cysts.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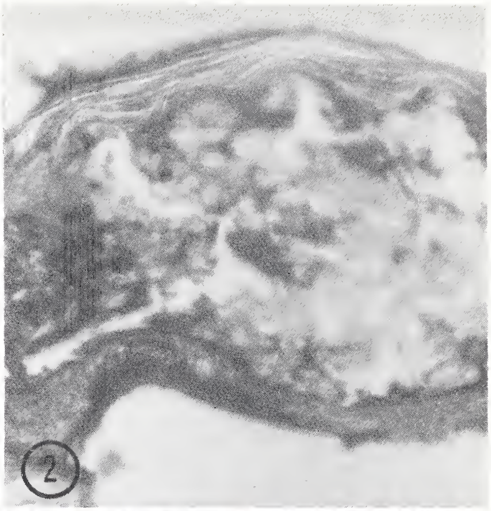
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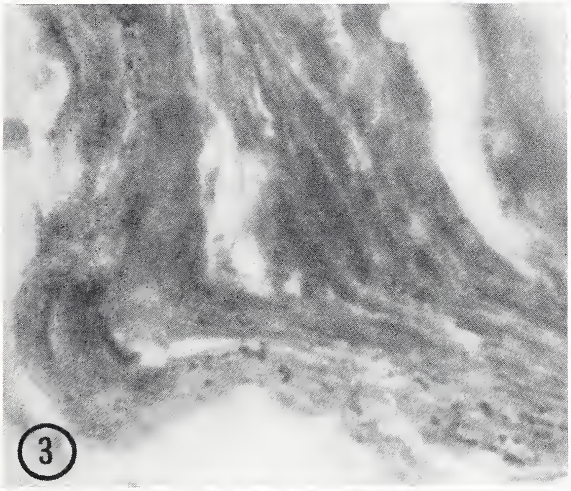
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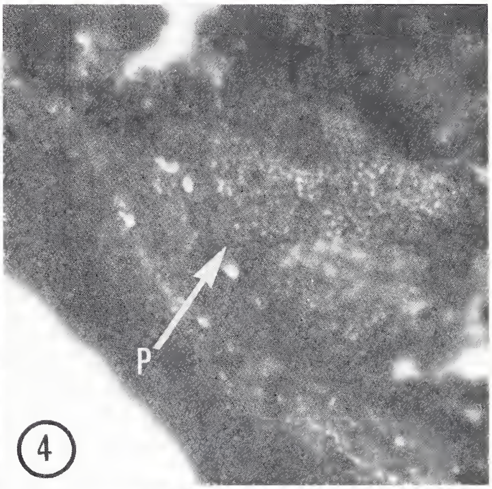
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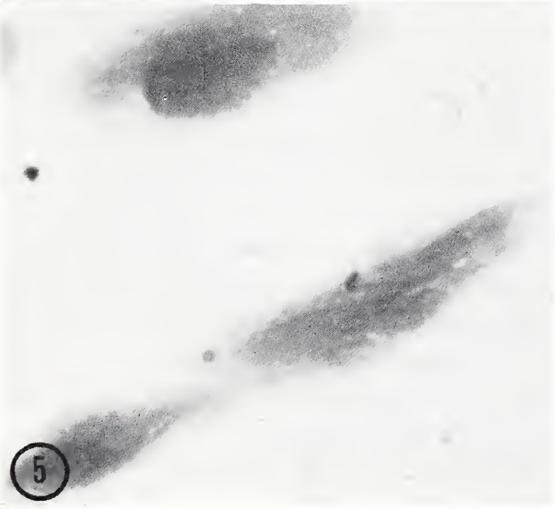
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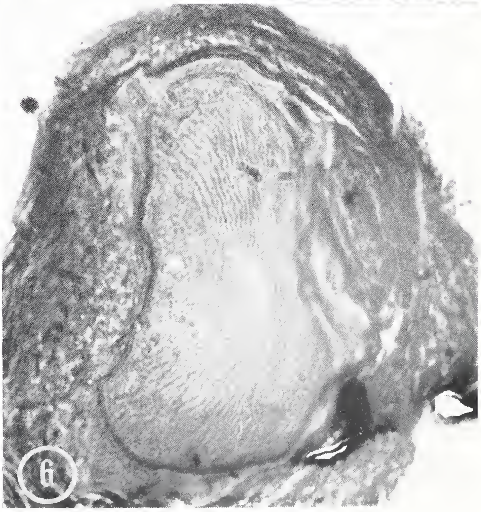
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Hemocytes and the Problem of Tumors in Insects [1]

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SUMMARY

A description is given of the various types of hemocytes in insects, and a uniform nomenclature for them is proposed. Current techniques for studying these cells are listed. It is suggested that abnormal or anomalous growths or accumulations would be far more accurate designations for insects than the misleading but widely used terms, tumors, cancers, and neoplasms. At the present time, no quantitative information is available on the hemocytes of even one species of insect which dies of one given anomalous growth.—Nat Cancer Inst Monogr 31: 481–485, 1969.

INSECTS DIFFER profoundly from vertebrates. To mention only a few of the differences, insects are covered with a segmented or jointed exoskeleton, possess a ventral nerve cord, and have a single, tubular, dorsal heart; most of them completely lack veins and arteries, and their blood has neither erythrocytes nor respiratory pigment (*1*). While all insects lack erythrocytes (or anything comparable to them), all possess leukocytes which are termed "hemocytes" (*2*).

Although various hemocytes of insects may superficially appear to resemble various vertebrate leukocytes, none have ever been profitably compared with any of the well-characterized types of vertebrate leukocytes.

At present, our knowledge concerning the existence of a compact tissue which generates, matures, and periodically releases circulating cells during the life of an insect is most unsatisfactory. In the few cases where such tissues are thought to exist (*3–6*), either the released cells generally rapidly fall out of circulation and are confined to tissue surfaces, or we have no information on how much the released cells contribute to the circulating population (*2*). Even where such organs are reported to be

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[2] I wish to acknowledge the suggestions and encouragement of Dr. John C. Harshbarger and Dr. Clyde J. Dawe.

present, it is well known that mitoses of hemocytes can normally take place in the circulating blood (*2*) and that maturation and marked transformations of hemocytes also occur there (*7*). Even though compact organs have been found which are stated as producing hemocytes in a few insects (*e.g.*, *Drosophila* larvae), these organs bear not the slightest resemblance to a vertebrate lymph node, and the still widely used term "lymph gland" ought to be exchanged for the more meaningful term "hemocytopoietic organ" (if this is indeed their function).

While examinations of blood cells are routinely and profitably used in the diagnosis of numerous diseases of vertebrates, with standard procedures and a commonly accepted terminology, we have yet to explore the value of such examinations in most insect diseases. Enough information has been accumulated, however, to suggest that, if a uniform terminology for the various types of hemocytes and our current methods for obtaining information on these cells are widely accepted, we can begin to determine the possible diagnostic value of hematologic examinations and find out the roles of hemocytes in various insect diseases.

The following uniform terminology (*2*) could be used to refer to the seven major types of hemocytes that can be found among insects: 1) *prohemocytes*, for small, round, intensely basophilic cells, generally the only type seen in mitotic division; 2) *plasmatocytes*, for highly pleomorphic cells (either round, ovoid, stellate, or fusiform with a single round or ovoid nucleus) which tend to form few to many pseudopodia and are often phagocytic; 3) *cystocytes*, for those cells which rapidly lyse in freshly removed blood (the cytoplasm rounds up and becomes intensely hyaline, and the round, cartwheel-like nucleus is often ejected into the plasma) and specifically cause the plasma to coagulate in their vicinity (not to be confused with cells in the germarium of the ovaries of *Drosophila*); 4) *granular hemocytes*, for those cells with generally many, small, discrete, granular eosinophilic inclusions often of uniform size; 5) *adipohemocytes*, for those cells often possessing large, spherical, refringent, fatlike inclusions or vacuoles and sometimes other heterogeneous granules; 6) *spherule cells*, for cells with many, generally very large, round, and/or ovoid, dense inclusions which tend to obscure the small nucleus; and 7) *oenocytoids*, for generally large, thick, often bizarrely shaped cells with conspicuously excentric nuclei. Their cytoplasm in some species may contain crystalline inclusions.

In a few insects, two other types of hemocytes have been described which have been seriously misunderstood by many investigators. These are the *podocytes* and the *vermiform cells*. True podocytes are exceedingly flattened stellate cells and are many times larger than the average plasmatocyte; indeed, their punctate nucleus alone is about the size of an average plasmatocyte. These cells do not send out pseudopodia; their very long, pointed cytoplasmic extensions do not retract *in vitro*. Unfortunately, many workers have referred to plasmatocytes, which happen to have sent out pseudopodial extensions, as podocytes. Plasmatocytes with pseudopodial extensions should be so designated and definitely should not be categorized as podocytes. Vermiform cells are extremely long, intensely basophilic, threadlike cells with a very thin elongated nucleus. When present in hemolymph, they are strikingly different from fusiform and elongated plasmatocytes, primarily in their great length and thinness. Fusiform and elongated plasmatocytes definitely should not be categorized as true vermiform cells.

The following techniques are currently used to obtain critical information on hemocytes (*2*):

Hemolymph (*blood*) *volumes* are made by either exsanguination, injection of such dyes as amaranth red or Evans blue, or injection of ^{14}C-inulin.

Hematocrits are obtained after centrifuging blood taken from living (unfixed) insects and from those which have been immersed in hot water (heat-fixed) to prevent both clumping of hemocytes and coagulation of the plasma. Heat fixation does not affect the blood volume.

Differential hemocyte counts are made on blood taken from living and heat-fixed insects by examination with phase contrast of either *a*) fresh, undiluted, wet coverslip mounts, or *b*) Versene-fixed wet coverslip mounts, and/or *c*) by making a thin smear of heat-fixed blood which, after being air-dried, is treated with either Wright or Giemsa or Mallory's triple stain. A series of sequential 100 cell counts are made, and usually a minimum of 200 cells must be counted (preferably more). Ideally, all three methods should be used because each one tends to give radically different images (especially stained smears). With some insects, glutaraldehyde-fixed material is extremely valuable and should be examined with phase contrast.

Total hemocyte counts (cells per μl of hemolymph) are made from both living and heat-fixed insects either by diluting a known volume of blood with acetic acid or Versene in a Thoma pipette, or by placing the blood directly on a hemocytometer chamber without dilution. Unfixed and heat-fixed samples give radically different values (unfixed counts are generally strikingly less than heat-fixed samples).

Histologic sections of whole insects or portions of them are made after placing the animals or their organs in cold or hot fixatives, and treating the tissues with a wide variety of stains, chiefly hematoxylin and eosin or Mallory's triple stain. Although this approach is indispensable to the pathologist, it often does not give a necessarily highly reliable picture of the types of hemocytes that may be present in abnormal growths.

Terminology concerned with anomalies of growth and form is a most complex problem. Peculiar difficulties are associated with the widely used term "tumor" with reference to a variety of abnormal growths and cellular accumulations among insects. "Tumor" has been used by some insect pathologists in its literal sense to mean any enlargement or swelling of a given tissue. Unfortunately, some insect pathologists have assumed that tissue enlargement from abnormal accumulations and/or abnormal multiplication of cells in insects with or without the subsequent death of the insects signifies that these phenomena are comparable to neoplasms or cancers of vertebrates. Death in normal and diseased animals can occur without any cellular growth. Abnormal enlargements of tissues without any cellular multiplication can readily cause death. Death can occur independently of very extensive cellular proliferation. Since our knowledge of vertebrate neoplasms so outweighs what we know about abnormal growths in insects, and since we still lack some of the most elementary quantitative information on even the simplest abnormal growth in insects, it would be most helpful if insect pathologists would refrain from the highly specialized terminologies of the vertebrate pathologists. Such terms as "cancer," "neoplasm," "leukemia," and "benign and malignant growths" for insects are confusing and misleading to the vertebrate pathologist. At present, it would be more accurate for the insect pathologist to use more noncommittal words such as "anomalous or abnormal growths" or "accumulations."

While there is no doubt that hemocytes of various insects can greatly increase in the circulating hemolymph in a number of diseased states, great increases can also occur in normal insects. Whereas hemocytes can infiltrate and destroy various tissues in diseased insects, in many insect orders (*e.g.*, Lepidoptera, Hymenoptera, and Diptera) the hemocytes normally destroy various old larval tissues. Although hemocytes are unquestionably involved in a number of anomalous growths associated with the subseqent death of *Schistocerca* (*8*, *9*), ephemerid larvae (*10*), *Pieris* (*11*), and *Drosophila* larvae (*12–15*), we have little or no information on either the types or numbers of hemocytes during any phase of the disease.

Some melanotic anomalous growths of *Drosophila* apparently arise from plasmatocytes or plasmatocyte-like cells, or are intimately associated with them; oenocytoids ("crystal cells") are also frequently seen in these growths. Whether other types of cells are involved is not known with certainty. Quite a number of other melanotic anomalous growths in *Drosophila* clearly do not arise from hemocytes or hemocyte-like cells. The extent to which hemocytes subsequently become associated with such growths has not been studied in detail. Abnormal growths of alimentary canal organs arising after nerve sectioning in various orthopteroid insects might well become infiltrated with hemocytes in the later stages of these anomalies. As yet, however, we have no information on the types or numbers involved. Since some hemocytes (*e.g.*, prohemocytes) are extremely primitive cells, they could easily be mistaken for other types of cells (*e.g.*, myoblasts). Because plasmatocytes are normally highly pleomorphic cells, they can readily simulate many other types of cells. Their ability to undergo marked transformations might readily be accentuated in pathological states. The fact that many different kinds of cells can dedifferentiate is bound to lead to much uncertainty in classifying the origin of cells in anomalous growths in insects. For example, it may actually prove impossible to decide whether a cell is a hemocyte or an epidermal cell. When such situations arise, it is worth recalling that assumptions are easy to make and difficult to prove.

I should like to conclude with the reminder that absolutely no *quantitative* information is available on the hemocytes of even one species of insect which actually dies of an anomalous growth. The adoption of a uniform terminology and methodology for studying hemocytes is clearly needed before we can begin the difficult task of assessing their roles in such diseases.

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Effect of Plant and Insect Hormones on the Formation of the Goldenrod Gall

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SUMMARY

The injection of various plant hormones such as indole-3-acetic acid, *p*-chlorophenoxyacetic acid, *N*-6-benzyladenine, and kinetin failed to elicit cecidogenesis or gall formation on goldenrod *Solidago canadensis* L. Butanol extracts of *Eurosta solidaginis* (Fitch), the goldenrod gall fly, do cause limited gall formation. Subsequent studies using ecdysterone and farnesyl methyl ether did not produce galls although this evidence is not conclusive.—Nat Cancer Inst Monogr 31: 487–491, 1969.

CECIDOGENESIS OR GALL formation by plants involves abnormal cell division which may be similar to abnormal cell division in some animal tumors. The round globose gall commonly found on the stem of goldenrod *Solidago canadensis* L. is caused by the pentration of a fly larvae, *Eurosta soligdaginis* (Fitch), into the meristematic tissue of the terminal bud. Division of the parenchyma cells ensues and the medulla becomes extended. Almost no enlargement of the cortex occurs and the cambium has approximately the same area of phloem and xylem cells as the normal stem. The morphology of the cecidium or gall has been studied by Ross (*1*) and our observations agree closely with this report.

Since it is apparent that the fly larva elicits some response in the plant to trigger abnormal cell division, the present investigation was initiated to elucidate what particular component or substance the insect released.

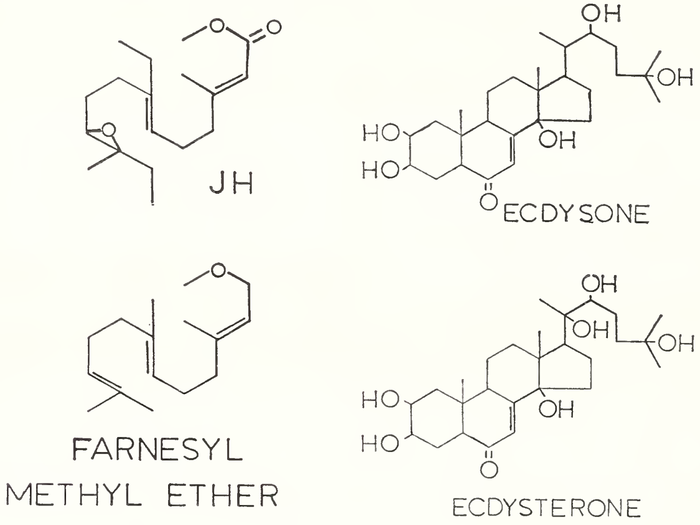
METHODS

Goldenrod was obtained from wild cultures in the field. The larvae of *Eurosta* were taken from galls obtained in the field or from goldenrod grown in the greenhouse and experimentally infected.

Saline extracts were prepared by homogenizing second or third instar larvae in 0.77 M NaCl (0.25 ml per animal) in a ground glass homogenizer. The extract was filtered through glass wool and centrifuged at approximately 7000 × *g* for 20 minutes. Butanol extracts were obtained as shown above except *N*-butanol was substituted for the saline and 0.5 ml per animal was used. The butanol layer was separated from the aqueous layer with a separatory funnel after centrifugation.

Ecdysterone and farnesyl methyl ether (text-fig. 1) were injected in 10% ethanol. Indole-3-acetic acid was made up in water and the other 3 plant hormones (*p*-chlorophenoxyacetic acid, *N*-6-benzyladenine, and kinetin) were dissolved in alkaline isopropyl alcohol-water. Injection was with a 10 or 50 μl syringe.

The soft fluid from the posterior portion of the alimentary canal was denoted as the feces and was diluted one-half with water before injection.



Text-figure 1.

RESULTS

Effects of Plant Hormones

Extracts of cecidogenic organisms (*2*, *3*) and plant growth substances (*3*, *4*) have produced galls after artificial application. It has been suggested that the insect causing the gall releases auxins within the plant (*5*). It is quite possible that *Eurosta* larvae either supply necessary precursors of indole-3-acetic acid (IAA) such as tryptophane or secrete a material which would stimulate IAA production. Thus, the first series of experiments were designed to elucidate the effect of IAA on goldenrod gall formation.

Concentrations of IAA varied from 10^{-3} M to 10^{-9} M and were applied as single or multiple injections. A differential growth rate was noted but no galls were formed. Several of the plants were used later for oviposition studies and subsequently produced galls.

Since IAA apparently has no effect, another auxin, parachlorophenoxyacetic acid was investigated. Similar experiments as shown above for IAA were conducted, but again, no evidence of gall formation was noted. Two kinins, *N*-furfuryladenine (Kinetin) and *N*-benzyladenine were also used in an attempt to cause gall formation but without success. The results of these experiments are summarized in table 1.

TABLE 1.—Effect of plant hormones on the incidence of gall formation*

Plant hormone	Concentration of hormone	Number of plants	Number of galls
Indole-3-acetic acid	1×10^{-3} M to 1×10^{-9} M	67	0
p-chlorophenoxyacetic acid	1×10^{-4}, 1×10^{-6}, and 1×10^{-8} M	6	0
Kinetin	1×10^{-3} M	3	0
	1×10^{-5} M	3	0
N-benzyladenine	1×10^{-3} M	3	0
	1×10^{-5} M	3	0
Natural oviposition	—	21	17

*All injections were both single and multiple (day 1, days: 1,3; 1,3,5; 1,3,5,7).

From these studies it was concluded that the individual plant hormones were not the causative substance. This, of course, does not preclude the supposition that the plant hormones may act together in some undetermined ratio.

Effect of Insect Extracts

The next line of approach involved the possibility that substances from the fly larvae were implicated in some way. Saline extracts were prepared from whole larvae homogenates and were injected into the primary meristem of the terminal bud. These experiments involved single and multiple injections of varying concentrations but in no case did the extracts elicit cecidogenesis.

Since the saline extracts were negative it was surmised that perhaps the terpene and/or sterol hormones were associated with gall formation and that these were not extracted with the aqueous solution. Butanol was subsequently used as the homogenizing medium and after centrifugation the water layer was removed via a separatory funnel. Injections equivalent to one-fifth of a larva produced galls in 3 of 28 cases. Butanol alone, at all concentrations, always gave negative results. The data from this series of experiments are shown in table 2.

Effect of Insect Hormones

The ability of the insect extract to produce gall formation suggested that the molting hormone, ecdyson, could be involved. Since ecdyson itself was not readily available, ecdysterone, a close analogue (text-fig. 1), was

Table 2.—Effect of fly larvae extracts on the incidence of gall formation

Type of extract	Concentration of extract	Number of plants	Number of galls
Saline	Equivalent of 1/10–2 larvae	47	0
Butanol	Equivalent of: 1/10 larva	15	0
	1/7.5 larva	28	0
	1/5 larva	28	3
	1/2 larva	28	1*
	1 larva	28	0
Butanol control	—	56	0
Feces	1–4 larvae	38	0
Natural oviposition	—	28	17

*This gall was only partially formed.

used instead. Ecdysterone has been shown to cause fly pupation (*6*, *7*) and recent experiments in this laboratory have shown that it will cause ecdysis in the American cockroach.

However, injection of the molting hormone had no effect at 1, 3, and 5 μg. Most of the experiments involved single injections but 6 multiple injection studies (5 μg for 4 days) were conducted. Unfortunately, the terminal bud was adversely affected by these treatments and control plants grew faster in the 21-day period than the experimentals. The results suggest that ecdysterone does not cause cecidogenesis, but a more comprehensive investigation is needed to supply conclusive proof.

Table 3.—Effect of insect hormones on the incidence of gall formation

Hormone	Amount of hormone injected	Number of plants	Number of galls
Ecdysterone	1, 3, or 5 μg	6	0
	5 μg per day for 4 days	6	0
Juvenile hormone	1, 3, or 5 μg	6	0
	3 μg per day for 3 days	3	0

Fly larvae have an additional hormone which is released in relatively high concentrations during early larval life. This substance, appropriately named juvenile hormone, inhibits the formation of adult characters by the larval form. The hormone has been isolated and identified (*8*) but, like ecdyson, is not readily available. Thus, a synthetic substitute, farnesyl methyl ether, was utilized. The similarity of the two compounds is shown in text-figure 1.

Several concentrations were employed both in single and multiple injections. However, no gall formation was observed in any experiment. It was concluded that juvenile hormone or more precisely a compound similar to juvenile hormone was not responsible for cecidogenesis.

DISCUSSION

The results obtained in this investigation are inconclusive. Injections of single compounds (both plant and insect hormones) fail to elicit cecidogenesis. Previous investigations [(*9*) ; Mills, unpublished] have shown that the larvae and not the egg are responsible for gall formation. Thus, it is not surprising to find that extraction of the whole larvae with an organic solvent such as butanol does produce a limited number of galls.

Since no single factor gives positive results, it appears that cecidogenesis is complex. Probably, one or more compounds are released from the insect, which in turn cause the secretion of several plant substances. Thus, the use of multiple hormone systems with various ratios of both insect and plant hormones may prove to be fruitful in subsequent experiments.

There is also the possibility that specific dermal glands of the insect integument may release a compound that would trigger the division of the parenchyma cells. A more detailed study of the insect's excretory products may yield positive results, although injection of urea, allantoin, and uric acid was without effect when injected in physiological concentrations (Mills, unpublished).

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Multiplication and Cytopathology of a Plant Tumor Virus in Insects[1]

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SUMMARY

The wound tumor virus (WTV) produces a systemic infection in various plant hosts and the formation of tumors and other abnormal forms of growth in a few species, such as crimson clover, sweet clover, and sorrel. Wounds seem to have a part in tumor initiation, the heredity of the host is important, and the hyperplastic tissue grows indefinitely if grafted from plant to plant. The virions are 60 mμ in diameter, icosahedral, and contain double-stranded RNA. The capsid consists of 92 capsomeres. In addition to susceptible plants, WTV also infects, and multiplies in, several species of agallian leafhoppers that act as alternate hosts and vectors of the virus. The multiplication in the leafhopper *Agallia constricta* has been demonstrated by serial passages, by an assay of soluble antigen, and by particle-counting methods. Virus assembly sites, so-called viroplasms, form in cells in which WTV multiplies. Sites of virus assembly were observed in the cell cytoplasm of the insect's fat body, salivary gland, hemocytes, gut, and nervous system. In the nervous system, WTV has been localized in ganglion cells, glial cells, perineurium cells, and tracheoblasts. Isolated virions were usually surrounded by polyribosomes and often appeared around foci of the viroplasm. These findings led to a search for possible deleterious effects of WTV on its invertebrate animal host. Electron micrographs of fat body and gut tissues of WTV-infected insects showed a cytological change in the fine structure. Degenerating ganglion cells infected with WTV strongly indicated that the virus exerted a cytopathic effect on the nerve cells of the invertebrate animal host. This double-stranded RNA virus causes cytopathic changes that are reminiscent of those induced by DNA viruses that produce experimental malignancies. The reported changes have been found only in *A. constricta* insect tissues *in vivo*. No cytopathic effects have been observed in tissue cultures of this vector species when the cells were infected with WTV *in vitro*.—Nat Cancer Inst Monogr 31: 493–507, 1969.

WOUND TUMOR VIRUS (WTV) is an ether-resistant icosahedral virus, 60 mμ in diameter, containing a double-stranded RNA. The virus causes an experimental disease in many species of plants belonging to dif-

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ferent families, but it has not been observed to cause disease in nature. It was recovered from one species of leafhoppers, collected in the vicinity of Washington, D.C., and was subsequently found to infect experimentally two additional, related species of leafhoppers. At present, the economic importance of WTV is unknown. As a result of certain unusual characteristics, WTV has attracted the attention of many workers in the relatively short time since it was first recognized. The production of abnormal growth in a few species of plants, the unusual double-helical RNA of high molecular weight, the multiplication in plants and in invertebrate animals, the resemblance to reoviruses of man and of a wide variety of lower animals, the multiplication in insect cells *in vitro*, the systemic infection of insect vectors, and the cytopathic effects in the vectors have been studied in great detail.

HISTORY

The term "wound tumor virus" was proposed by Black (*1*) for the causative agent of a clover disease, discovered by him and first called clover big vein disease (*2*). Black found that the virus produces a systemic infection in many plant hosts and irregular vein enlargement and tumors in some. In sorrel plants, *Rumex acetosa* L., wartlike enations are formed on leaves, and roots develop numerous tumors. Root and stem tumors are formed in certain inbred lines of sweet clover plants, *Melilotus alba* Desr. and *M. officinalis* L. Lam. (fig. 1).

CLASSIFICATION AND NOMENCLATURE

According to the classification of viruses of the International Committee on Nomenclature of Viruses (*3, 4*), WTV belongs to the same group of double-stranded RNA viruses as reoviruses. The relationship between WTV and reoviruses is supported by the identity or close similarity of the viruses in size, shape, number and arrangement of capsomeres, double-stranded RNA, and the guanine-cytosine/adenine-uracil base ratios of RNA (*5*). Recently, it was found that the capsid of WTV appears to consist of two distinct layers that are not equally affected by physical and chemical agents, and that can be clearly distinguished by electron microscopy of partially degraded virus (*6*). This finding implies that more than one type of protein is present in the capsid. Similar findings have been made with reoviruses (*7*).

It has been reported that WTV and reoviruses might be antigenically related (*8*) but the data were not considered conclusive by the authors because no homologous titers were available; the preparations of reovirus had a very low titer, and the normal serum was pooled and no preimmune serum was used. Gamez *et al.* (*5*) failed to find serologic cross-reactivity in passive hemagglutination tests. Such tests, although quite sensitive, react only with precipitating antibodies, so that the possible existence of cross-reacting nonprecipitating antibodies could not be investigated by its application. Gomatos and Tamm (*9*) found no cross-reactivity between reoviruses and WTV, but again, as pointed out by Rosen (*10*), their data were not convincing. In the first instance, it was not demonstrated that the reactions observed were specific; in the second instance, it was not established beyond question that the WTV antiserum that failed to react with the reovirus antigens did indeed contain antibody against WTV. No virus replication was obtained when reovirus strains were inoculated into *A. constricta* Van Duzee leafhoppers capable of transmitting WTV (*10*). Purified WTV did not agglutinate human erythrocytes. Inoculation of monkey and mouse fibroblast cell cultures with WTV produced no cytopathic effects, nor were pathogenic effects observed in mice or hamsters inoculated subcutaneously or intranasally (*11*). When newborn mice were given injections of WTV preparations, tumors were sometimes observed a few months later, but a careful study by Streissle (unpublished data) indicated that these tumors occurred at a frequency similar to that of spontaneous tumors in the noninjected controls.

Several other plant-pathogenic viruses sharing some characteristics with WTV may be classified with it. The rice dwarf virus has a double-stranded RNA (*12–14*) and it replicates in the cytoplasm and not in the nucleus of cells (*15–18*). The maize rough dwarf virus, also transmitted biologically by its vector (*19*), probably belongs to the same group. WTV and rice dwarf virus have been shown to pass transovarially by infected female leafhoppers to a varying percentage of their progeny (*20*), but they are not transmitted through the sperm of viruliferous males when the latter are mated to virus-free females. Probably the restriction of these viruses to the cytoplasm is responsible for the lack of sperm transmission.

It is not certain whether the similarities between WTV, rice dwarf, maize rough dwarf, and reoviruses are due to a phylogenetic relationship or whether they are the result of parallel evolution from different phylogenetic sources (*4*, *5*).

PROPERTIES OF THE VIRION

The structure of WTV has been studied extensively by electron microscopy, with negative staining. Bils and Hall (*21*) estimated the diameter to be approximately 60 mμ. The same values were obtained by Shikata and Maramorosch (*17*) who measured the virions in a cross section of a microcrystal, formed naturally in the fat body cytoplasm of a leafhopper vector. The capsid contains 92 capsomeres (*21*), 7.5 mμ in diameter, numbering 4 along the edge, with smaller units of capsomeres of about 2.5 mμ regularly packed.

The chemical composition has been studied by Black and Markham (*22*) and by Gomatos and Tamm (*9*). The RNA content of the virus was reported to be about 20% as indicated by determination of the nucleic acid and protein content, ultraviolet absorption curve, and specific volume of the virus. The width of the RNA was visualized by electron microscopy (*23*).

HOST RANGE

In 1945, 43 plants of 20 different families were listed as susceptible to WTV infection (*1*). Obviously, many more plant species could be found to be infected if additional surveys of other plants were carried out (*24*). Among insect species, *A. constricta*, *A. quadripunctata* (Provancher), and *Agalliopsis novella* (Say) are the only three agallian species of leafhoppers known to be susceptible to infection. A few related species have been tested but failed to transmit the virus. If additional species were tested, probably some would be found susceptible to WTV.

PATHOLOGY

Plant hosts.—WTV produces enlarged veins and tumors on the roots of several plant species. In some plants, leaves curl and become distorted, stems thicken, flowers are suppressed, and the plants are dwarfed. However, no clearing (chlorosis) of veins occurs as a primary sign. Some plant species, although infected, are almost indistinguishable from healthy ones, while others are severely affected and some are killed. The most pronounced tumors are in sorrel, *R. acetosa*, and in sweet clover, *M. alba* and *M. officinalis*. According to Lee (*25*) root tumors originate mainly at the base of emerging lateral roots. The intervening cells between the meristematic tumor cells and the endodermal layer eventually become tumorous so that in later stages the tumor is in direct contact with the endodermis. The first signs of tumor development appear at the fourth or fifth incipient lateral root behind the tip of the mother root at the time the lateral root is emerging. In old root tumors, groups of abnormal tracheids were found (*25*), encircled by parenchymatous tumor cells and by a few necrotic cells. Lee (*26*) also determined that there are many tumor initial cells scattered in young stems of systemically infected sweet clover plants, but that comparatively few of these develop into macroscopic tumors. In old tumors, occasionally slight intrusions of tumor tissue into xylem occur.

In many plant species infected with WTV Littau and Black (*27*) found spherical cytoplasmic inclusions called spherules. These spherules vary in number in different tumors and different cells, and occur most frequently in cells of the pseudophloem. No spherules were found in healthy plants. Spherules vary in size from 4 to 10 μ. Hyperplastic cells, formed in diseased crimson clover, often become necrotic (*28*). In an electron microscopy study of wound tumor neoplasia, Shikata and Maramorosch (*17*) found clusters of virions and scattered virions in necrotic and non-necrotic portions of tumor cell cytoplasm, but not in epidermal cells of tumors nor in the cortex of roots. Virion clusters enclosed in membranous structures were found in some stem tumor cells. They were abundant in root tumor cells near tracheids. Virions were absent from nuclei, chloroplasts, and mitochondria. Occasionally tubular structures were observed to be open at both ends and to contain strings of virions. The authors pointed out that virions were found only in the cytoplasm and not in the nucleus of abnormal cells. The multiplying virus nevertheless disturbs the normal growth-controlling mechanism and thus produces the effects that culminate in the formation of plant tumors. Perhaps the viral nucleic acid plays its neoplasm-inducing role before the virions are formed, and subsequent detection of WTV at any particular site might not be relevant to the neoplastic processes. It is conceivable that WTV transforms some normal cell constituent into a tumor-producing agent.

Wounds play an important role in the initiation of tumors (*29*). Tumors can be started in infected sweet clover stems by puncturing them with pins. The younger the punctured tissue, the greater the proportion of developing tumors. Cuttings from diseased plants usually develop tumors at the cut surface.

Heredity of the host is important in tumor development (*30*). Heredity influences the number, size, shape, distribution, and surface character of the neoplasms. Hyperplastic tissue can be grafted from plant to plant, and it retains its neoplastic characteristics indefinitely.

Invertebrate hosts.—WTV infects and multiplies in certain agallian leafhoppers that act as transmitters of the virus to plants. Maramorosch (*31*) showed that nymphs, as well as adult males and females, can transmit the virus. Plants can also be infected mechanically by multiple pin punctures through drops of virus-containing extracts, but this method is very inefficient (*32*). When insects are allowed to acquire virus by feeding on diseased plants, a minimum incubation period of 13–15 days elapses before they are rendered infective (*31*). Insects can also be rendered infective mechanically, by needle inoculation (*33*), specifically by the injection of WTV-containing extracts from diseased plants, or from infective insects. The injection method provided the first bioassay for WTV. Serological tests, developed by Black and co-workers (*34*, *35*), were more rapid and more sensitive than infectivity tests. Black (*24*) cautioned that serological tests cannot make the distinction between antigens to virions, noninfective virus particles, or virus protein.

VIRUS INVASION OF INSECT VECTORS

An electron microscopy study of thin sections prepared from viruliferous *A. constricta* permitted the precise localization of virions in the cytoplasm of fat body tissues, malpighian tubules, epidermis, trachea, muscle, mycetome, gut, salivary glands (*17*, *36*, *37*), the nervous system (*38*), and certain types of hemocytes (*39*). Serology indicated that the virus might also be present in the ovaries (*40*); WTV occasionally passes transovarially to the progeny of infected females and this vertical transmission can be increased considerably by selection and breeding (*41*).

The largest accumulations of WTV, often in the form of microcrystals, were found in the fat body tissues. Crystalline arrangements were also seen in muscle cells and in the gut (*17*) and, rarely, in the salivary glands (Shikata, unpublished data). Microcrystals were also formed in hemocytes (*39*). Virus clusters were observed in defined cytoplasmic structures within cells of various organs. Surprisingly, only one or two lobes of the salivary gland contained WTV (*17*). In the central nervous system, virions were mostly found in the cytoplasm of the ganglion cells and less frequently in glial cells. Occasionally, virions were also observed in the perineurium cells, nerve axons, tracheoblasts, and lateral nerves. In the ganglion and glial cells, virions appeared as individual isolated particles, in tubular formation, or in aggregates. In the perineurium cells and tracheoblasts only small virus accumulations were present. Isolated virions, usually surrounded with polyribosomes, were detected around foci of virus assembly (*38*).

MULTIPLICATION IN INSECT VECTORS

Multiplication of the virus in the vector *A. constricta* was tested by Black and Brakke (*42*), who applied the insect injection technique, carrying out 7 passages. Titration at each passage showed that there was no decrease in virus titer, and that there was as much virus present in the 7th group of insects as there was in the original source. Throughout the serial passages, the insects were maintained on alfalfa plants (*Medicago sativa* L.), which are not susceptible to WTV infection.

Serological tests, carried out by Whitcomb and Black (*35*), provided further evidence for the increase in virus titer in insects given injections of crude extracts of WTV.

Multiplication was also demonstrated by an electron microscopy examination of thin sections of insects that acquired the virus by feeding or by injection (*43*, *44*). In insects infected orally, viroplasms, *i.e.*, sites of virus assembly, were detected in the gut, fat body, salivary glands, and several other organs. The finding of viroplasms in hemocytes (*39*) indicated that virus multiplication occurs in the hemocytes which thus act not only as a carrier of the virus to various sites, but also as a source of fresh virus. Finally, virus assembly sites were found in the central nervous system (*38*).

CYTOPATHIC LESIONS

Nervous system.—In the ganglia, most of the ganglion cell bodies are situated in the periphery. Outer glial cells situated beneath the perineurium surround the ganglion cell bodies. Synapses were observed between ganglion cells and outer glial cells. Synaptic vesicles were seen in the synaptic region of the axoplasm. A healthy nerve cell is illustrated in figure 2. Occasionally, irregular inclusion bodies, without virions, were observed in the cytoplasm of ganglion cells. However, in most infected cells no significant changes in the fine structure of the cytoplasmic organelles were found, except in degenerating ganglion cells (*38*). Such cells were encountered in insects that carried WTV for several weeks. The cytoplasm of degenerating ganglion cells had a high electron density in comparison with that of normal ganglion cells (fig. 3).

The changes in the ultrastructure were often very pronounced. The cytoplasmic matrix was transformed into homogeneous fine granules. The membrane structures of cytoplasmic organelles, such as the endoplasmic reticulum, the nuclear membrane, and the Golgi apparatus, were indistinct. No normal mitochondria were found in the cytoplasm. The ribosomes of the cell were loosely coagulated. The nuclear substance was also changed to a homogeneous fine granular substance. A number of virions, either isolated or in tubular formation, were clearly observed in the cytoplasm of these degenerating ganglion cells.

The degeneration of ganglion cells appeared to be very severe in some instances, but no direct deleterious effects or behavioral disorders were observed in virus-transmitting *A. constricta.* Although it has not been established whether WTV causes a disease *sensu strictu* of the invertebrate host, these degenerating ganglion cells demonstrated a cytopathic effect on the nerve cells.

Cytopathic lesions in the fat body cells.—When *A. constricta* were given injections of virus extracts, no pronounced cytological changes occurred within the first week after inoculation (*44*). A normal-appearing fat body cell is shown in figure 4. Ten days after inoculation with WTV, remarkable cytopathic changes were commonly observed in a limited part of the fat body. The cytoplasm appeared loose and abnormal, indicating deterioration of the fine structure (fig. 5). Whenever this type of cytopathic change was detected, virions were also found in these, or in adjacent cells. No changes of this type were ever found in fat body cells of virus-free controls given injections of extracts from healthy insects or healthy plants.

The above-described cytopathic changes were different from those that occurred in older insects irrespective of virus injection. Contrary to the typical cytopathic changes found only in WTV carriers, a nonspecific vacuolation was observed in old insects. The vacuolation was more common in WTV carriers but it was also seen in controls.

Cytopathic changes in the gut.—The cytoplasm of uninfected gut cells contains a large number of ribosomes, scattered Golgi areas, and typical metazoan mitochondria (fig. 6). *A. constricta* leafhoppers which acquired WTV by feeding on diseased plants showed cytopathic changes in the epithelial cells of the filter chamber, which is a part of the midintestine. The cytologic changes first appeared during the week after WTV acquisition (fig. 7). In infected cells of the midgut, the ultrastructural integrity of the cytoplasm was profoundly changed due to depletion of ribosomes, to disarrangement of endoplasmic reticulum, and to degenerations of mitochondria. The overall electron opacity of the infected cell was decreased due to a decrease in the number of ribosomes and to formation of cytoplasmic vacuoles. Nuclei were often abnormally swollen.

INTERACTION WITH ARTHROPOD CELLS *IN VITRO*

The culture *in vitro* of *Homoptera* tissues and cells has been successful only within the past 10 years (*45*). The development of monolayer cultures (*46–48*) of fibroblast-type and epithelial-type cells, derived from embryos, or from nymphal and imaginal tissues (*49*), permitted the rational application of invertebrate tissue culture to the study of WTV. It was hoped that plaque formation would allow quantitative studies but this goal has not yet been achieved. WTV replicates in cells of its insect vector *A. constricta*, but produces no cytopathic effects in such cell cultures (*50*). The growth rate of infected cells is comparable with that of healthy lines, indicating that WTV-infected cells are apparently not impaired by the multiplication of the virus. In the absence of visible plaques, virus proliferation had to be demonstrated by the immunofluorescent cell count technique (*51*). It was calculated that the insect injection technique (*33*) can detect WTV concentrations in the range of $10^{2.6}$ (*52*). Whenever WTV concentration in cells *in vitro* exceeds $10^{2.6}$, infectivity tests can thus be applied for bioassay.

From indirect calculations of the protracted incubation period of WTV in insect vectors and from the slow increase in virus titer in vector cells *in vitro*, it can be assumed that WTV has a relatively long reproductive cycle. This is reminiscent of the long reproductive cycle of reoviruses (*53*), and is probably the function of the double-helical nucleic acid (*10*).

DISCUSSION AND CONCLUSIONS

Although WTV produces abnormal growth in a few species of plants, in many other plants the infection does not result in the formation of neoplasms. In the invertebrate animals that act as alternate hosts and carriers of the virus, the systemic infection and WTV proliferation cause no tumors, and cytopathic changes have only been found in the fine structure of certain cells. These cytopathic changes in fat body, ganglion, and gut cells are of particular interest. They are reminiscent of those induced, as a rule, by all deoxyribonucleic acid viruses that produce experimental malignancies, although WTV contains a double-stranded RNA. Oncogenic RNA viruses, as a rule, have not been found to exert cytocidal effects on cells. They either multiply without any morphological effects or they produce malignant transformation (*54*).

Cytopathic changes have not been detected in vector cells *in vitro*, whereas they have been demonstrated in cells *in vivo*. However, the changes described by us have only been detected by electron microscopy of the fine structure, and they were not apparent under phase-contrast or light microscopy. Besides, neither fat body nor ganglion cells have been grown *in vitro*, and it is not known whether embryonic cultures used for WTV contained gut cells.

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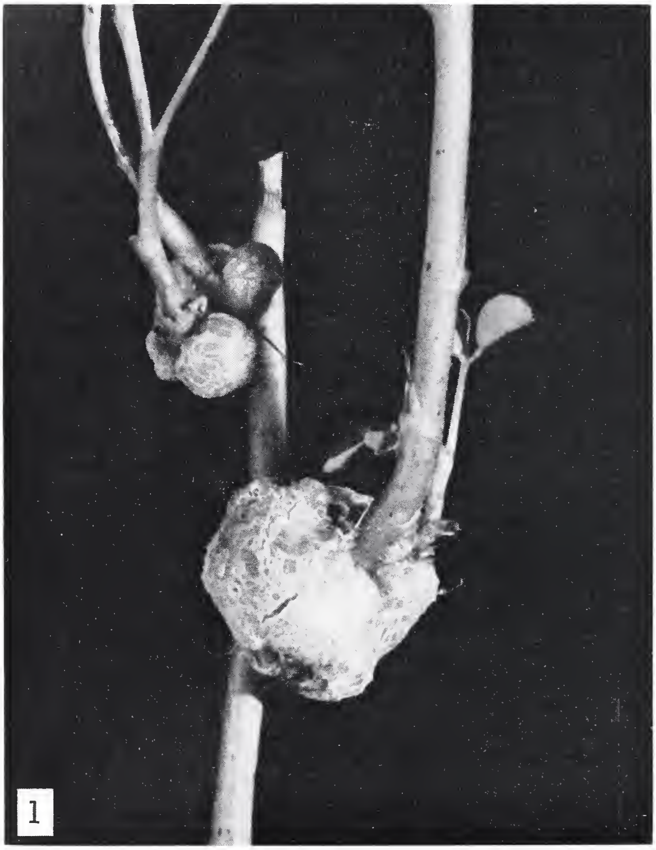
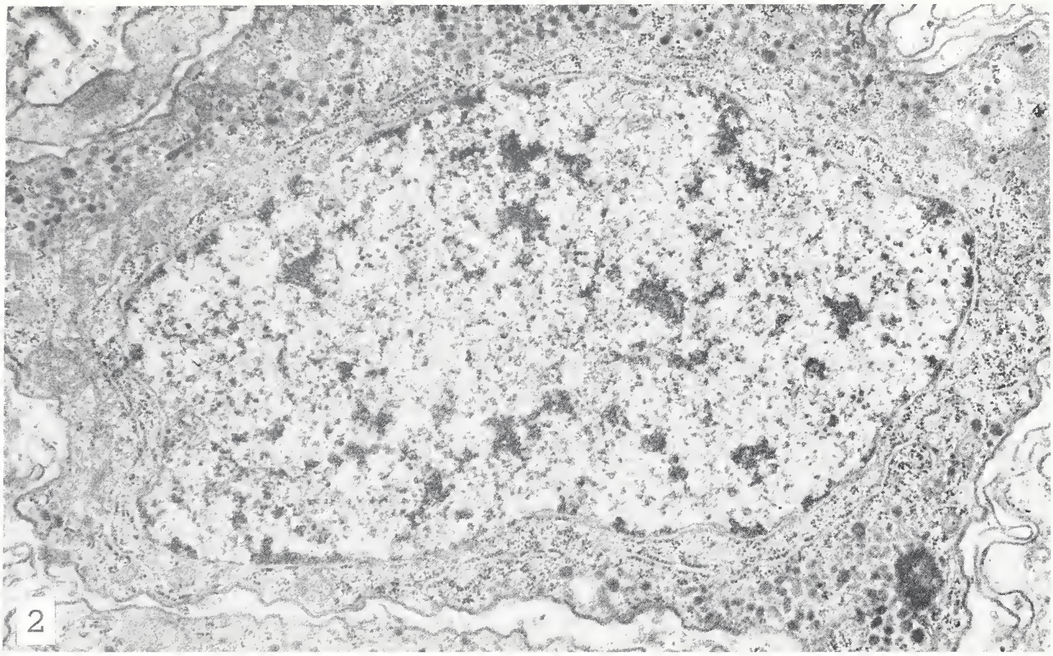


FIGURE 1.—Stem tumers on *M. officinalis* plant infected with WTV. × 3



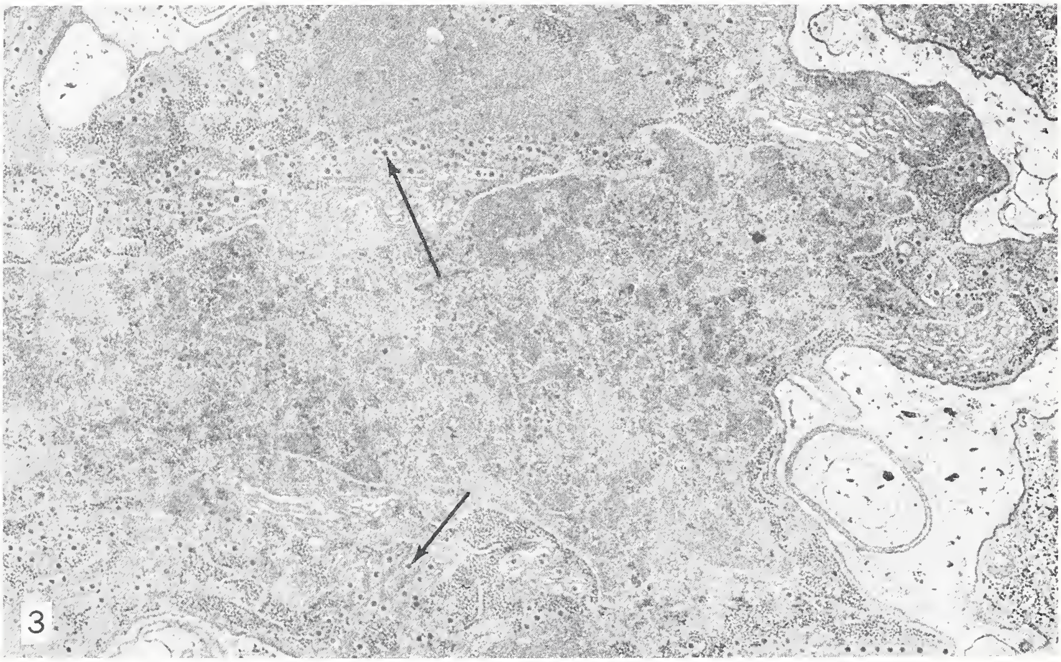
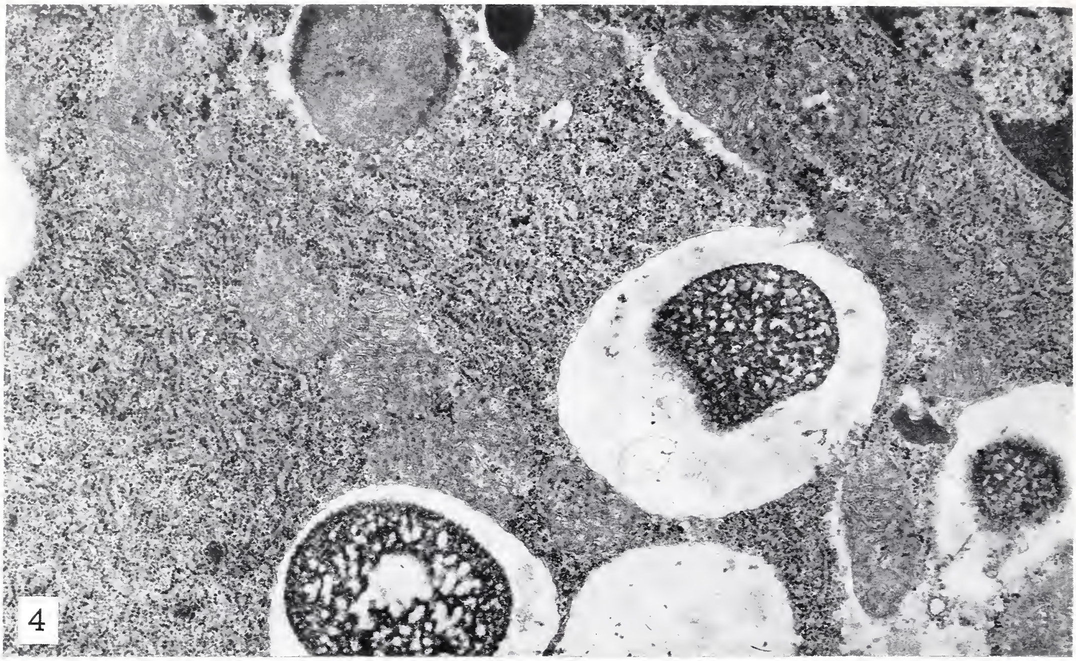


FIGURE 2.—Healthy nerve cell in prothoracic ganglion of *A. constricta.* × 20,000
FIGURE 3.—Cytopathic effects, induced by WTV (*arrows*) in degenerating nerve cell in prothoracic ganglion of viruliferous *A. constricta.* × 21,000



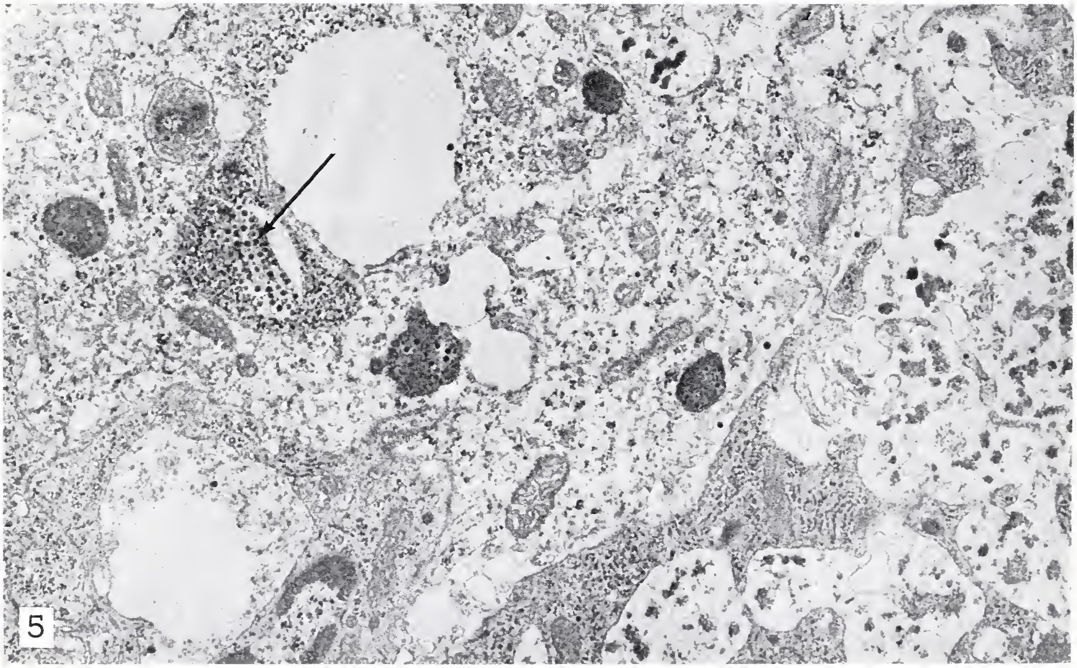
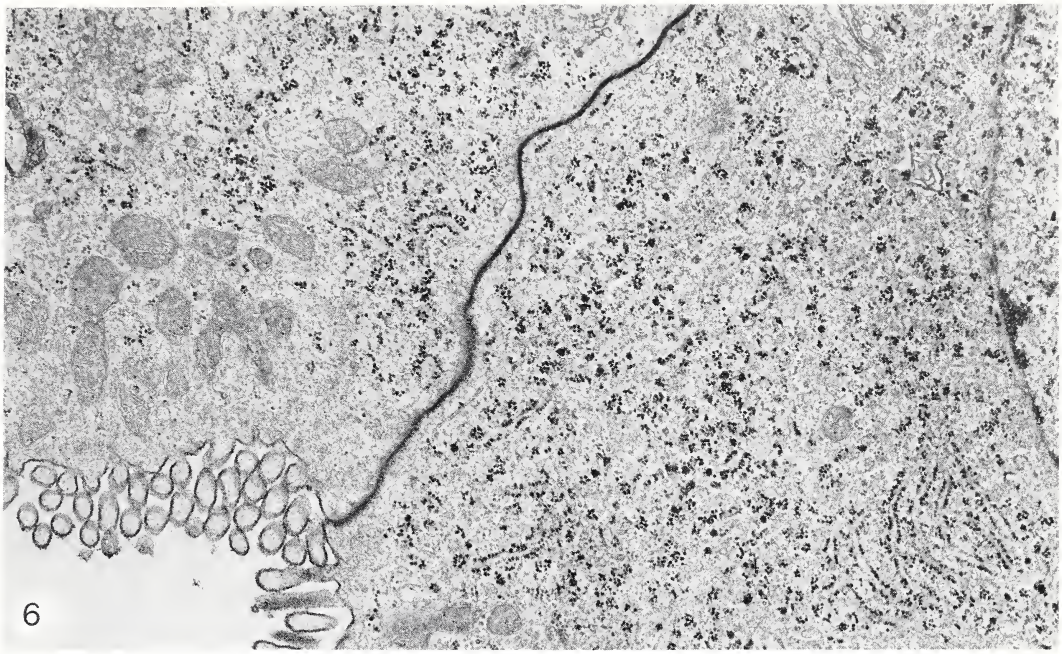


FIGURE 4.—Portion of healthy fat body cell of *A. constricta.* × 15,000

FIGURE 5.—Portion of degenerating fat body cell of viruliferous *A. constricta. Note* cytopathic effects induced by WTV (*arrow*). × 12,000



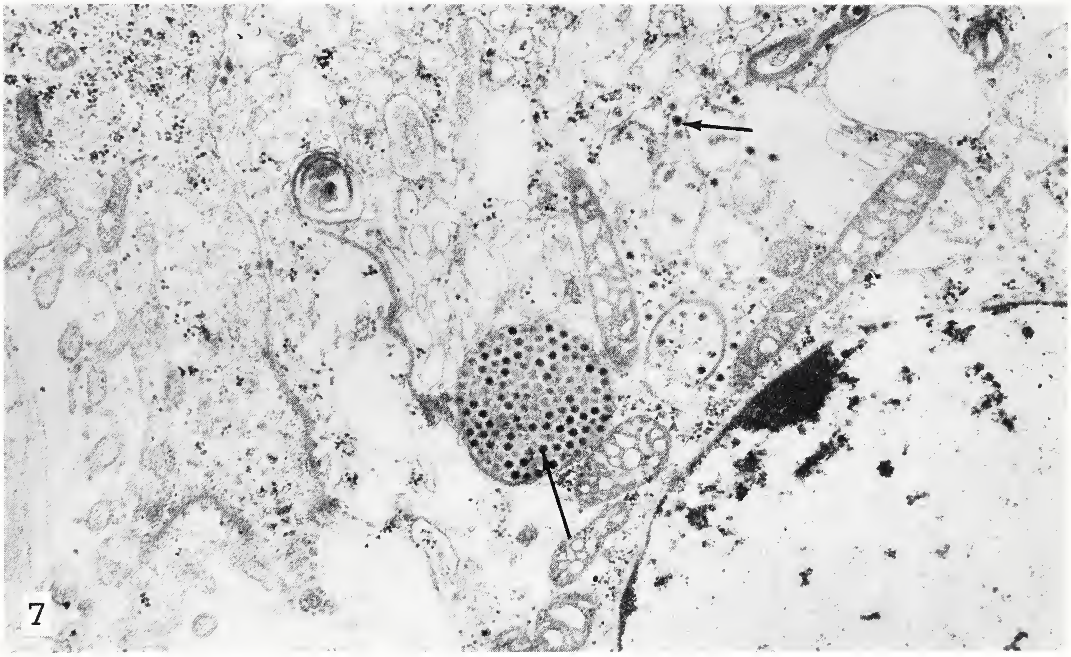


FIGURE 6.—Healthy epithelial cells in midgut of *A. constricta.* × 24,000

FIGURE 7.—Portion of a degenerating epithelial cell in midgut of a viruliferous *A. constricta*, showing cytopathic effects induced by WTV (*arrows*). × 30,000

A Critical Review of Neoplasia and Tumor-Like Lesions in Mollusks[1]

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SUMMARY

A critical review of the literature on molluscan neoplasia and tumor-like lesions is presented, including some previously unpublished data on the prevalence of tumors in freshwater and marine pelecypod populations. Neoplasms have been reported from the three classes of mollusks of economic importance (Pelecypoda, Gastropoda, and Cephalopoda). However, among mollusks, as in many other invertebrates, the distinction between neoplasia, hyperplasia, and response to injury or infection is often uncertain, because no decisive criteria currently exist to aid the invertebrate pathologist in the diagnosis of neoplasia among these animals. Therefore, the author sets forth criteria for determining uncontrolled growth (neoplasia) in mollusks, to aid in distinguishing neoplastic growth from other cellular responses. Previously unpublished descriptions of lesions on the gills of *Anodonta californiensis* and on the foot of *Gonidea angulata* are presented.—Nat Cancer Inst Monogr 31: 509–539, 1969.

SPONTANEOUS NEOPLASMS have been observed in animals of many of the invertebrate phyla. Some of these reports have been isolated and casual observations incidental to a primary study (*7*, *37*, *58–60*), while others are incomplete or contain erroneous descriptions of the lesions (*19*, *30*, *51*). Invertebrate oncology, excluding that of insects, has received very little attention. Indicative of the dearth of research on invertebrate neoplasia is the fact that most pertinent reviews on the subject were published over a decade ago (*15*, *48*, *52*, *70*, *71*).

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Sinderman and Rosenfield (*54*) have briefly mentioned tumors in their review of the principal diseases of commercially important marine bivalve mollusks and crustaceans. However, this was not an extensive review of the literature on molluscan tumors. Although neoplasms may have an inimical effect on individual mollusks, these reviewers were primarily interested in diseases that are capable of decimating shellfish populations.

The neglect of oncologic data concerning mollusks is regrettable because *a*) mollusks are ubiquitous and easy to collect and examine; *b*) they are easy to maintain in the laboratory, as they can withstand large variations in environmental conditions without adverse effect; and *c*) apparently they can develop true neoplasms. However, the paucity of information about neoplasms of mollusks and other invertebrates makes the distinction between neoplasia, hyperplasia, and response to injury or infection difficult to determine confidently, mainly because there are no trenchant established criteria to diagnose neoplasia in these animals. This indicates the need for better understanding of these processes in mollusks and other invertebrates—an understanding that will be achieved only after careful examination of a large number of naturally occurring and induced tumors. Zoologists familar with invertebrate habits, anatomy, and histology are often unfamiliar or unconcerned with pathologic principles and do not realize what a significant part disease plays in the life and ecology of invertebrate fauna. Conversely, pathologists studying neoplasia among vertebrates have little or no opportunity to study invertebrates and rarely realize that the simplicity of the molluscan body offers an excellent system in which to study the mechanisms of neoplasia.

LITERATURE REVIEW

Inflammatory and Parasitic Lesions

In some cases gross lesions observed were thought to be neoplastic until histologic examination revealed them to be inflammatory responses or parasitic infestations. Growths do exist that are difficult to classify as either reactive or neoplastic. Naturally occurring lesions of this sort are discussed under "Neoplasia."

Pelecypoda

The consideration has been expressed (*60*) that neoplasms in bivalves may be the result of injury or irritation and are not true neoplasms as the word is used in vertebrate pathology. However, recent studies on wound reactions and injury responses in oysters and other mollusks indicate that these animals react similarly to vertebrates after injury (*44–46*, *68*, *69*) and these responses are dissimilar to those in neoplasia. For example, metaplastic changes from tall columnar to low cuboidal or squamous epithelium have been observed in the gut of the oyster, *Crassostrea gigas*, and attributed to trauma caused by the parasite, *Mytilicola orientalis* (*57*).

Taylor and Smith (*65*) reported polypoid and papillary lesions on the foot of the gaper clam, *Tresus nuttalli*, with gross characteristics so suggestive of neoplasia (fig. 1) that only histologic examination of the lesions revealed them to be inflammation and hyperplasia (fig. 2). Their highly cellular character made the differential diagnosis between neoplasia and hyperplasia difficult, an observation stressed earlier by Scharrer and Lochhead (*52*) in their review of invertebrate tumors. Taylor and Smith (*65*) observed one large (4 × 7 mm) and three smaller polypoid lesions (1 mm or 1.5 mm in diameter), all of which were firm, fleshy, and of the same color as the rest of the foot. Histologic examination of these lesions revealed an intense inflammatory reaction with a heavy infiltration of both hemocytes and fibroblasts. Microorganisms were present in some of the phagocytic cells. However, it was not known if these microorganisms were the cause of the lesions or only secondary invaders.

A previously undescribed protozoan parasite, *Haplosporidium tumefacientis*, was found to be the cause of small tumefactions (fig. 3) in the digestive gland and kidney of California sea mussels, *Mytilus californianus* (*64*). One thousand bay mussels, *M. edulis*, and 1,114 sea mussels, *M. californianus*, were examined and 2.1% of the latter were found to have enlargements of the digestive gland and kidney resulting from plasmodial infection of these organs.

Pauley (*39*) recently examined 75 freshwater mussels, *Margaritifera margaritifera*, that had resilient watery lesions. Histologically, they were edematous or collagenous lesions that the author thought were evoked by an unidentifiable microorganism.

Gastropoda

A snail, *Arion ater*, with a humped back was noted by Boettger (*1*). He observed atrophy of the mucous glands and hyperplasia of the connective tissue of the skin. Within the connective tissue and the muscle just beneath it were numerous cysts, which were thought to be gregarine parasites. Boettger thought the hyperplastic reaction was evoked both by the mechanical presence of the parasites and by a chemical produced by the invaders.

Michelson (*32*) observed prominent swollen tentacle tips on the snail, *Helisoma anceps*. Histological examination revealed that the tentacles were infected by an acid-fast bacteria, *Mycobacterium* sp., and the swellings were the result of the host's response to the pathogens. White "cyst-like" nodules on the reproductive organs of aquatic pulmonate snails belonging to the family Physidae were the result of a microsporidian parasite, *Plistophora husseyi*, replacing the normal snail tissue (*33*).

A moon snail, *Polinices lewisii*, was observed with large nodular, edematous lesions in the foot (*55*). Although the resilient growths resembled neoplasms upon gross examination (fig. 4), microscopic examination revealed them to be edematous lesions containing scattered muscle fibers and several different microorganisms. These tumor-like swellings apparently developed in response to the microorganisms, which were found throughout the snail's body. In many respects these lesions resembled the "spongy" growths observed on freshwater mussels, *M. margaritifera* (*39*). Blister-like swellings have also been observed by Richards (*50*) on the foot, head, tentacles, and mantle collar of three species of snails (*Australorbis glabratus*, *Tropicorbis riisei*, *Tropicorbis pallidus*) infected by amebae, *Acanthamoeba* sp. Hypertrophy of individual host cells was caused by one or more amebae within each cell of the blister. The disease was easily transmitted to uninfected snails (*50*). However, other attempts to infect gastropods with *Acanthamoeba* sp. have been unsuccessful (*12*).

Neoplasia

Although five classes of mollusks exist, reports of neoplasia have been confined to the three economically important groups: Pelecypoda, the Gastropoda, and the Cephalopoda. Since no trenchant criteria exist in mollusks for distinguishing between hyperplasia and neoplasia, some of the reports mentioned in this section undoubtedly refer to hyperplastic responses.

Pelecypoda

Small polypoid growths were reported on *M. margaritifera*, measuring 2–3 mm in diameter and 2 mm in height (*39*). All growths were firm, normal in color, and attached to the foot by a small stalk. The surface was covered by normal ciliated, tall columnar epithelium. The growths were composed almost entirely of an increased number of normal-appearing basophilic gland cells, with a few supporting muscle and collagen fibers. No mitotic figures were present and the lesions were not invasive. There was no inflammatory reaction associated with these lesions, which were thought to be due to hyperplasia of the basophilic gland cells.

A freshwater mussel, *Anodonta californiensis*, was reported to have a small, firm, tumor-like growth on the foot that measured 3 mm in diameter and 2 mm in height (*35*). The growth consisted of dark basophilic glandular cells and muscle fibers and had a deeply convoluted epithelial covering. It was classified as an adenomyoma because of its muscular and glandular composition. However, it was pointed out that it was possibly unwise to name this growth by applying terminology developed for vertebrate pathology. The epithelial cells of this lesion had pale-staining, vacuolated cytoplasm different from that of the normal epithelium. The basophilic gland cells and muscle fibers were arranged irregularly. Glycogen is the reserve material of oysters (*10*) and other mollusks and is stored primarily in the connective tissue or Leydig cells. The distribution of glycogen in the tumor-like growth on *A. californiensis* showed no apparent abormality. There was no evidence of invasion into the surrounding normal tissue, and the lesion was considered to be a benign neoplasm or a hyperplasia of the basophilic and muscle cells beneath the foot epithelium. The cause of the lesion was not known, but if it was a hyperplasia, it may have been produced by some type of irritation or injury.

Williams (*72*) examined 700 freshwater mussels, *Anodonta cygnea*, and observed one benign, ovoid, pedunculated tumor arising from the mantle, approximately the size of a hazelnut. Microscopically, the lesion consisted of gland cells and a muscular core, all of which were arranged in an abnormal fashion. Unfortunately no drawings are available to illustrate the position of the tumor.

Collinge (*4*) examined approximately 300 freshwater mussels, *A. cygnea*, and found two growths which he interpreted as neoplasms. One was similar to that observed by Williams (*72*), except that it was not attached to the mantle by a stalk. The other growth was an elongated mass located on the mantle near the dorsal siphon and connected with both the mantle and the outer gill. The mantle is the organ in mollusks that is responsible for secretion of the shell (*10*). There was an indication that this lesion had seriously interfered with the normal physiology of the mussel because of a dearth of nacreous material on the posterior valves and the loose calcareous layer of shell. Unfortunately there are no drawings to illustrate the position of these lesions, and no measurements or microscopic descriptions of either of these two growths, so it is difficult to compare them with other molluscan tumors.

Butros (*2*) described a large nodular neoplasm that measured 2.0 cm in length and 0.8 cm in diameter arising from one of the inner labial palps of the freshwater mussel, *A. implicata.* The tumor was covered by a columnar epithelium that was smaller and more irregular than normal. Connective tissue cells were very abundant near the periphery of the neoplasm but scanty in the center of it. The connective tissue cells were larger and more granular than those of the palps. In the tumor there was an increased number of hemolymph sinuses filled with hemocytes. The author did not discuss the nature of this lesion but it was apparently benign. The occurrence of a few mitotic figures was noted. Evidence that mitotic figures have rarely been observed in mollusks was given by Hillman (*17*).

Four freshwater mussels, *A. californiensis*, with pedunculated growths arising from the foot were reported by Pauley (*37*). Of 40 mussels examined, these 4 had a total of 14 benign tumors, all arising from the foot. The polypoid growths were elevated from the surface of the foot by firm but flexible stalks. The lesions varied in size from 1 × 2 × 2 mm to 4 × 4 × 2 mm, with 2–6 tumors per animal. The growths were all covered by a convoluted, tall, columnar epithelium. There was a thick, dense layer of basophilic gland cells beneath the epithelium and a central core of compacted muscle fibers. No mitotic figures were observed and no inflammatory reaction was present. Pauley (*37*) termed these pedunculated adenomas, but another observer thought their nature probably hyperplastic (*6*)

Several populations of freshwater mussels were examined for tumors (*40*). The lowest incidence observed was 4.0% (12 of 300 animals) and the highest was 28.4% (2 of 7 animals). Table 1 summarizes the tumor incidence data for four species of freshwater mussels collected from the six sites indicated in text-figure 1. The tumors ranged in size from 0.5 mm–10 mm in diameter, and most were observed on the foot (figs. 5–9). The histological appearance of the lesions examined was similar to that previously reported (*37*). Among the *A. californiensis* population from the Columbia River, two mussels were found with small hyperplastic lesions on the mantle and two animals had large edematous growths on the gills (figs. 8 and 9). Tumors arising from the gills have not previously been recorded for mollusks. Multiple tumors were observed on twelve of the *A. californiensis* and on one *Gonidea angulata.*

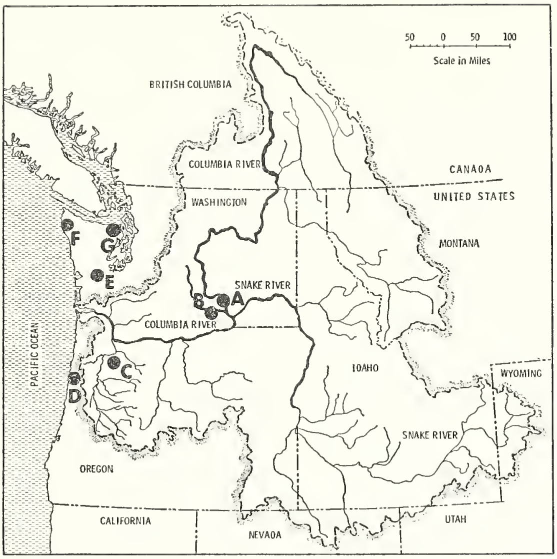
TABLE 1.—Incidence of tumors in various freshwater mussel populations

Collection site	Species	Number of mollusks examined	Number of animals with tumors	Number of tumors observed	Animals with tumors (%)
Ozette River, Washington	*Margaritifera margaritifera*	123	8	8	6. 5
Chehalis River, Washington	*M. margaritifera*	71	4	4	5. 6
Mollala River, Oregon	*M. margaritifera*	300	12	12	4. 0
Devil's Lake, Oregon	*Anodonta oregonensis*	388	17	17	4. 2
Columbia River, Washington	*A. californiensis*	299	47	78	15. 7
Yakima River, Washington	*Gonidea angulata*	7	2	3	28. 4

Histologically, the lesions on the gills of *A. californiensis* did not appear to be neoplastic (fig. 10). They were hyperplastic folds of normal gill tissue that were edematous and heavily infiltrated with hemocytes.

One freshwater mussel, *Gonidea angulata*, from the Yakima River had two tumor-like growths on the foot. The larger was 5 × 4 × 3 mm (fig. 11) and the smaller 3 × 2 × 2 mm (fig. 11). The large lesion (fig. 12) appeared to originate from the smooth muscle but only traces of muscle fibers were present in the lesion. The growth consisted mainly of collagen, as demonstrated by a modification of Mallory's collagen stain (*36*). There was edema, an incomplete epithelial covering, and a hyperplasia of the gland cells. This growth may have resulted from chronic irritation, as indicated by the abundant collagenous stroma, the incomplete epithelial covering, and the hyperplastic gland cells. On the other hand, it may have started as a polypoid neoplasm and the collagen was the effect of chronic irritation to the polyp. The smaller lesion (fig. 13) apparently had its origin in the smooth muscle of the foot. The lesion was edematous and covered by a highly convoluted epithelium. Neither lesion invaded normal tissue or possessed mitotic figures.

Hueper (*19*) reported cauliflower-like papillary tumors around the rectal opening of about 2% of a sample of soft-shell clams (*Mya arenaria*)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Text-figure 1.—Location of marine and freshwater Pelecypoda collection sites in the Pacific Northwest. *A: Anodonta californiensis* (Columbia River). *B: Gonidea angulata* (Yakima River). *C: Margaritifera margaritifera* (Mollala River). *D: A. oregonensis* (Devil's Lake). *E: M. margaritifera* (Chehalis River). *F: M. margaritifera* (Ozette River). *G:* Sequim Bay is the collection site of all marine species listed in table 2.

collected from Chesapeake Bay, near Thomas Point. He did not state the total number of clams examined or the number with tumors. Although photographs of the gross lesions were presented, no microscopic descriptions were given.

Investigators at the Laboratory of Pathology of the National Cancer Institute later examined 500 *M. arenaria* from the Thomas Point area, where Hueper's specimens had been taken, and 500 from an area near Solomons, Maryland, without discovering any additional papillomas (*3*). Sections of one of Hueper's papilloma-bearing clams have been accessioned by the Registry of Tumors of Lower Animals (Accession No. 115. *See* RTLA Activities Report, 1968, J. C. Harshbarger, p 33). These, in conjunction with Hueper's published gross photographs, show that the lesions were noninvasive papillary proliferations of mantle epithelium at the pedal orifice of the mantle, not near the rectum.

Hillman (*18*) found an *M. arenaria* with a large tumorous mass protruding from the mantle (fig. 14), but histologic examination failed to reveal its nature. The lesion contained sand, which may have stimulated or induced the lesion, or it may have been there incidental to the primary tumor.

A cylindrical growth measuring 1.3 × 4.0 mm was reported on the siphon of a soft-shell clam, *M. arenaria*, by Potter and Kuff (*49*). The structure had a firm, muscular consistency similar to that of the siphons.

It was made up of normal smooth muscle arranged in inner longitudinal and outer circular layers as in the normal siphon and was covered by normal epithelium.

Five hundred soft-shell clams, *M. arenaria*, were collected from the Tred Avon River estuary at Oxford, Maryland, in 1965 (*41*). One animal had a fungiform, wrinkled swelling at the basal portion of the siphons that measured 12 × 9 × 6 mm (fig. 15). The major portion of the growth was located ventrally and along the right side of the siphons. Microscopically, the tumor was almost entirely covered by highly convoluted columnar epithelium possessing deep folds or crypts, except for a few ulcerated areas. The stroma appeared to be entirely smooth muscle which was confluent with the smooth muscle of the siphons from which the tumor originated. The neoplasm appeared to have two distinct lobes separated by a cleft that extended into the lumen of the siphon. Each lobe of the tumor was attached to the ventral side of the siphon by a thin stalk. There was moderate hemocytic infiltration throughout most of the lesion, especially noticeable in the ulcerated areas. There were also pockets or nests of inflammatory cells. Edema was present just beneath the surface epithelium in many places. The lesion was apparently benign, since it was composed entirely of normal tissue that showed no sign of invasiveness and had no mitotic figures.

A similar tumor of smooth-muscle origin was noted on the foot of one of 183 butter clams, *Saxidomus giganteus*, examined by Pauley (*38*). It was a large polypoid tumor measuring 9 × 6 mm (fig. 16). The growth was firm and similar to the normal foot in both texture and color. Microscopic examination of the tumor revealed the entire growth was covered by a somewhat convoluted, tall, columnar epithelium that resembled the normal epithelium. The major portion of the tumor was composed of smooth muscle, which appeared to originate from the circular layer of smooth muscle just beneath the epithelium of the foot (fig. 17). It did not appear to be a response to injury and was considered to be a benign tumor that lacked mitotic figures.

A similar but smaller finger-like growth was found among 394 *S. giganteus* from Sequim Bay, Washington, that were examined subsequently (Pauley, unpublished data). The growth was firm and had the same color and texture as the rest of the foot and measured 2.5 × 1 mm. It was covered by tall, columnar epithelium that was somewhat hypertrophied and hyperplastic. The core was composed of smooth muscles, originating from the circular layer of muscle, and no mitotic figures were observed. Table 2 summarizes the tumor prevalence data for six species of marine bivalves collected from Sequim Bay, Washington (Pauley, unpublished data). For some unknown reason there is a disparity of tumor prevalence between freshwater (table 1) and marine pelecypods (table 2).

A large, soft, pliable, nodular tumor measuring 1½ inches long by 1 inch wide was observed in the pericardial cavity of an oyster, *C. virginica*, by Ryder (*51*). The pericardial cavity, which lies just anterior to the single large adductor muscle, was enlarged because the tumor had displaced the

TABLE 2.—Shellfish collected and examined for tumors from Sequim Bay, Washington, and its vicinity

Species of mollusk	March 1967		June 1967		Total	
	Number collected	Number of tumors	Number collected	Number of tumors	Number collected	Number of tumors
Schizothaerus nuttalli (Gaper clam)	49	0	10	0	59	0
Clinocardium nuttalli (Basket cockle)	30	0	28	0	58	0
Mytilus edulis (Bay mussel)	77	0	295	0	372	0
M. californianus (California sea-mussel)	484	0	—	—	484	0
Saxidomus giganteus (Butter clam)	183	1	394	1	577	2
Protothaca staminea (Common Littleneck clam)	253	0	—	—	253	0

heart forward. This tumor appears to have originated ventrally from the connective tissue surrounding the rectum, or dorsally from the lining of the pericardium. The rectum passes dorsally over the heart in oysters (*10*, *53*). Although there is no illustration of the tumor, a vivid picture of it can be formed in the reader's mind, because of the lucid description of the gross lesion.

The histologic structure of this tumor has been misinterpreted by several authors (*2*, *43*, *56*, *59*) because of the statement by Smith (*56*) that: "The tumor described in this paper [Smith's] is not unlike the one found by Ryder." A cursory reading of Ryder's (*51*) description leads one to believe that the tumor he described in *C. virginica* was a connective tissue tumor of undetermined origin that had burst or ruptured the integument lining the pericardium. If that were true, the tumor described by Ryder (*51*) and Smith (*56*) would be very similar, but I do not think they were alike. Ryder (*51*) stated that there was not an integument over the surface of the tumor, while Smith (*56*) found a very distinct epithelial covering of tall, columnar cells on the tumor he described. The confusion among subsequent investigators regarding the nature of the tumor described by Ryder would probably have been avoided if some illustrations of the tumor had been included.

Smith (*56*) described a nodular tumor in *C. virginica*, measuring 1¼ × 1 × ⅓ inches, that was connected to the outer surface of the pericardium by a narrow, delicate stalk. The tumor and its stalk were composed of Leydig cells covered by a single layer of ciliated columnar cells. The tumor cells contained numerous, finely dissolved lipid granules that do not appear to differ from those found in normal Leydig cells (*10*). This was a benign lesion, with an intense inflammatory reaction just beneath the surface epithelium.

Sparks *et al.* (*59*, *60*) noted two similar tumors on Pacific oysters, *C. gigas* (figs. 18 and 19). One of these lesions was a stalked, ovoid, mesenchymal tumor (fig. 18) measuring 20 × 15 mm, located just dorsal to the rectum (*59*). The neoplasm was connected to the body in the area of the rectum, from which it originated. Microscopically, the tumor consisted of a stroma of relatively typical Leydig cells, but it contained an increased amount of collagen, particularly in the center of the tumor and underlying the basement membrane of the epithelial covering. The Leydig cells in the center of the neoplasm contained conspicuous amounts of glycogen, while those at the periphery lacked glycogen, and the normal Leydig cells found throughout the oyster possessed large amounts of glycogen (Sparks and Pauley, unpublished data). There was an extremely heavy hemocytic infiltration beneath the epithelium of the tumor. The tumor was covered mainly by convoluted, tall, columnar epithelial cells, similar to those found on the free edges of the mantle.

On gross examination, the other oyster appeared to have an identical lesion (fig. 19), measuring 24 × 18 × 16 mm. However, microscopic examination revealed that the apparent tumor was actually the expanded rectum, evidently obstructed by fecal impaction (*60*). Mackin (*31*) discussed a number of structural abnormalities apparently associated with infections by microorganisms, but neither he nor other investigators have reported a condition comparable to the tumor-like fecal impaction. The authors (*60*) observed a similar abnormality in the intestine of an oyster, *C. virginica*, included among sections of Smith's (*56*) mesenchymal tumor loaned to them by Dr. Victor L. Loosanoff. However, the intestinal affliction in *C. virginica* was not readily apparent because it was not lying externally on the body. Microscopic examination of the *C. gigas* lesion showed it to be a laminated noncellular mass with a granular central part that was eosinophilic and contained colonies of bacteria and fungi scattered through it. The adjacent epithelium, thought to be that of the rectum, was not compressed by the large fecal mass, but the tissue directly beneath the basement membrane was flattened and appeared to be forming a fibrous wall. This specimen reemphasizes the importance of a detailed histologic examination to evaluate the nature of tumorous lesions.

A second mesenchymal tumor, with an irregular, nodular surface, was noted by Sparks *et al.* (*59*) in *C. gigas* (figs. 20 and 21). The neoplasm was stalked and crescent-shaped, and measured 30 × 13 × 15–20 mm. It was lateral and anterior to the adductor muscle, covered the pericardial area, and had protruded through the left side of the mantle. It originated ventrally from the main body mass, grew into the suprabranchial chamber, penetrated the mantle, and then grew dorsally. The tumor was examined microscopically in five areas, each of which was somewhat different in histologic structure. In one place there were cryptlike depressions on the tumor surface lined by tall, columnar epithelium, but the major portion was smooth and covered by a single layer of cuboidal epithelium. In a second area the neoplasm was made up of Leydig cells, heavy collagen deposits, and edematous areas near the surface. In this area the tumor had several concentric layers of muscle and collagen fibers, with finer fibers running perpendicular to the concentric layers. There were structures in the tumor that appeared to be digestive tubules under examination at low magnification, but were in reality rings of collagen enclosing what appeared to be hemocytes. These structures were named "pseudo-tubules" (fig. 22). The third and fourth sections resembled the second. At the periphery of the fifth area were normal Leydig cells with pseudo-tubules and a highly edematous central area with a few scattered Leydig cells. No mitotic figures were observed, and the lesion was considered benign.

Pauley and Sayce (*42*) noted two tumors from oysters, *C. gigas*, that were improperly fixed, making microscopic examination of the lesions unsatisfactory. One of the oysters had a smooth-textured, dark-green growth arising from the inside of the mantle, measuring 17 × 13 × 10 mm (fig. 24). The other oyster had a bifurcated adductor muscle, with an apparent polypoid or papillary neoplasm growing from the abnormal anterior portion of the muscle, measuring 25 × 10 × 10 mm (fig. 23).

Tumors on mollusks are usually discovered because they are large, readily visible masses, protruding externally on the surface of the animal. For this reason, there have been only three descriptions of internal tumors in mollusks, all in oysters (*29*, *43*, *47*).

One lesion (*43*) was palpable as a hard mass on the dorsal surface of the animal (*C. gigas*). It had a hollow center and appeared to be encased in a fibrous capsule. It was rather large, measuring 52 × 19 × 23 mm, and had displaced much of the normal tissue. Microscopically, the tumor consisted of a stroma of fibrous connective tissue and collagen (fig. 25), with a few normal Leydig cells present. The fibrous connective tissue cells all had round nuclei, but many of the cells were elongated. The neoplasm was perforated by sinusoids containing hemocytes. The tumor appeared to originate from undifferentiated gonadal cells and had many tubular structures present at the edge of it that appeared to be immature gametogenic tubules.

Because of their tendency to be spindle-shaped with long, bipolar, cytoplasmic extensions, the tumor cells resembled smooth muscle. The lack of a glandular pattern ruled out their possible origin from the digestive tubules which were in contact with the tumor at a few points. Periodic acid-Schiff (PAS) stains with and without diastase showed that normal Leydig cells adjacent to the tumor were filled with glycogen, while those within the tumor had very little glycogen. The normal Leydig cells surrounding the tumor contained an abnormally high number of brown-granuled "pigment cells," similar to those described by Stein and Mackin (*61*), that occur at times of stress and disease. The tumor border was well defined, with the tumorous mass apparently pushing the normal tissue aside rather than invading it. The expansion of the tumor had flattened some of the cells adjacent to it. No mitotic figures were observed, and the neoplasm was considered benign.

Katkansky (*29*) recently observed two small polypoid growths in the intestine of a European oyster, *Ostrea edulis*. One growth was mushroom-shaped, measuring 0.15 × 0.12 mm, and was supported by a stalk 0.04 mm in diameter (fig. 26). The growth consisted of slightly modified epithelial cells. A moderate hemocytic infiltration of the lesion was evident. The other lesion, measuring 0.15 × 0.20 mm, lacked a well-defined stalk and possessed a more prominent hemocytic infiltration. Both growths were opposite the typhlosole and were noninvasive.

An oyster, *C. gigas*, with multiple watery cysts, had an unusual proliferation of tissue infiltrating and virtually replacing areas normally occupied by Leydig cells and digestive tubules (*47*). The growth appeared to be a unique internal tumor composed of collagen and nervous tissue, accompanied by a marked inflammatory reaction in many places. Heavy deposits of collagen were closely associated with the nervous elements, but did not always form encapsulating sheaths around the nerves, such as are normally found in *C. gigas*. However, in many instances abnormally thick collagen capsules surrounded the nerves. There was an increased number of "pigment cells" similar to those described by Stein and Mackin (*61*). The association of the tumorous tissue with the watery cysts may have been coincidental, or there may have been some direct relationship between them.

This neoplasm appeared to be invasive, but nerves and ganglia are normally found within other tissues and organs—a fact that makes recognition of invasiveness of neural tumors difficult (*6*).

Tumors arising from the mantles of Sydney rock oysters, *C. commercialis*, have been observed by Wolf (*73*). Gross examination showed the polypoid growths had nodular, cauliflower-like characteristics (fig. 27) and were possibly of glandular origin. Histologically, the lesions were epithelial and almost identical to each other. The epithelial covering of the tumors was a mixture of ciliated, columnar epithelium and mucous cells. The epithelial cells comprising the major portion of the tumors (fig. 28) varied from tall columnar to cuboidal and there were very few mucous cells among them. There was an intense inflammatory response around and within the tumors. The lesions were invasive and infiltrated the pallial muscles and Leydig cells. Tumor cells had not penetrated or involved the outer mantle epithelium. However, the inner mantle epithelium was confluent with the neoplastic epithelium on the surfaces of the tumors. The lesions had numerous mitotic figures (figs. 29 and 30), most of which were in prophase or metaphase, but a few were in anaphase or in telophase. Hillman (*17*) has indicated that mitoses are rarely observed in bivalves. Mitotic figures in molluscan tumors are rare and have been reported only by Butros (*2*), Couch (*5*), and Farley (*7*). Despite their invasive activities, the tumors described by Wolf (*73*) showed a rather high degree of morphological organization and relatively little, though definite, cellular atypicality.

An oyster, *C. virginica*, examined by Couch (*5*) had a neoplasm in the mantle composed of large, basophilic, blastoid cells with pleomorphic nuclei. The high number of mitoses observed was atypical for normal oyster cells.

Farley (*7*) described a unique disease in oysters, *C. virginica* (5 cases) and *C. gigas* (1 case), that was characterized by massive proliferation of the hyaline hemocytes. Mitotic figures were extremely common, and it was thought that all the cases were hematopoietic neoplasms, though possible origin from primordial germ cells was discussed.

Gastropoda

The growth of a white, pedunculated, papillary tumor measuring 15 × 5 × 8 mm (fig. 31) on the mantle of a slug, *Limax flavus*, was observed over a 3-month period, during which time it proliferated rapidly, with alternating periods of decline and sloughing (*63*). Microscopically, the structure was covered by cuboidal or flattened epithelium, supported by muscle and connective tissue cells, and was glandular with an alveolar or tubular appearance. The alveoli were lined by a single layer of either columnar, cuboidal, or flattened epithelial cells. The histologic structure of the tumor in no way resembled any normal tissue in this gastropod.

Gersch (*11*) described a large tumor in a snail, *Helix* sp., that had filled approximately one-third of the lung. No measurement of the gross lesion was given, so that the actual size of the tumor is not known. Histologically, the tumor was composed of a proliferation of the Leydig cells, in which there was a reduction of the cytoplasm-to-nucleus ratio. The tumor replaced the normal respiratory epithelium and adjacent hemolymph spaces with a solid mass of connective tissue (Leydig) cells. Gersch (*11*) stated that this tumor must have seriously interfered with the normal breathing process of the animal, because of the large amount of respiratory surface that had been replaced or dislocated by the lesion, and concluded that the "formation must be labeled a tumor." This neoplasm appeared very similar to the benign mesenchymal tumors found in oysters (*56*, *58*, *59*).

Fischer (*8*) described a pedunculated fibrous tumor arising from the mantle of a gastropod, *Pleurobranchus plumula* (fig. 32). The growth was stalked, firm, and nonretractile with a complete epithelial covering. The tumor substance was composed of connective tissue cells and fibers of connective tissue origin (fig. 32). The tumor was highly vascularized and apparently benign.

Frömming *et al.* (9) reported on three gastropods possessing tumor-like growths; one was a humpback swelling on *Deroceras reticulatum*, and two were on the mantles of two different *Arion rufus*. Histologic examination revealed the growths on *A. rufus* resembled neoplasia, while the humpback on *D. reticulatum* was thought to be a congenital deformity.

Two similar flaplike tumors were observed on the posterior half of the mantle shield of *A. rufus* (*9*). The smaller of the two tumors was a small vesicular swelling which was smooth and glossy. The larger tumor was an ulcerated lesion. Unfortunately no measurements of the size of these two lesions and no photographs of the gross lesions were presented. The smaller tumor had pushed apart the layers of muscle beneath the epithelium and crowded out the intestine; it consisted of enormously enlarged (hypertrophied) mucous cells. The surrounding connective tissue and the tumor contained an intense inflammatory infiltration. The larger tumor on *A. rufus* caused a noticeable compression of the muscle layers and was very similar to the smaller neoplasm, consisting of hypertrophied mucous cells. Although the border between the tumor and the connective tissue was not sharp, there was no indication of infiltration. Frömming *et al.* (*9*) thought that both specimens of *A. rufus* had a tumor-like hyperplasia of the mucous membrane cells. These lesions differed only in their size and were thought to be different stages of very similar growths. Neither contained mitotic figures and both were considered benign.

Nolte (*34*) described a nodular tumor on the dart sac that was approximately 1 cm^3 in size and a similar, but smaller, tumor on the lower part of the albumin gland in one snail (*Helix pomatia*). Histologically, the tumors consisted of round vesicles (cysts) of varying size that were cellular proliferations within the connective tissue. The vesicles were often surrounded by a thin layer of muscle. In the albumin-gland tumor, some of the normal glandular tissue of this organ had been displaced and compressed. There was a tendency of the cells in the vesicles of the tumors to be spindle-shaped and segregated into distinct layers around a necrotic focus, indicating a well-organized hemocytic reaction. However, Nolte (*34*) diagnosed these lesions as definitely not inflammatory reactions, because there were no bacteria or parasites observable in them, but as lesions similar to the tumor in *Helix* sp. described by Gersch (*11*).

CEPHALOPODA

Jullien and Jullien (*25*) found two cuttlefish with tumors—one with five and the other with four. These were elevated, hard, whitish nodules that measured several mm in diameter on the ventral part of the visceral sac. These tumors appeared to be connective tissue neoplasms that were highly vascularized at the periphery. There were inflammatory reactions in the outer portions of the tumors and in the surrounding normal musculature. The connective tissue had lost its normal stratified appearance and was compact and homogeneous within the tumors. There were many elongate fibroblasts adjacent to the tumors and some within the tumors' compacted connective tissue. The neoplasms had no epithelial covering or chromatophores which was the reason they were white. The cause of these benign connective tissue lesions was unknown.

Effects of carcinogens.—Harshbarger (*15*) briefly reviewed the responses of invertebrates to vertebrate carcinogens, and it is indeed surprising to find how seldom mollusks have had experimental lesions produced in them. Although carcinogenic chemicals have been used by several investigators, irradiation has not reportedly been used on mollusks in an attempt to produce neoplasms.

PELECYPODA

Haranghy (*13*) studied the effect of benzo[*a*]pyrene on four species of freshwater mussels—*Dreissena polymorpha*, *A. cygnea*, *Unio pictorum*, and *U. tumidus*. He injected the carcinogen into the visceral mass of these animals, but was interested in the physiological effect of the chemical rather than its ability to induce neoplastic change in the normal tissue. Consequently there is no discussion of the microscopic appearance of the tissue. He noted that benzo[*a*]pyrene significantly reduced the ability of all four species to make clear water that had been made turbid by starch and carmine. The cilia are responsible for the ability of pelecypods to clear turbidity from their ambient water (*10*). It was therefore assumed that benzo[*a*]pyrene injections exerted a powerfully deleterious influence on the ciliary activity of freshwater mussels, and thus adversely affected the entire metabolism of these animals (*13*).

GASTROPODA

Tripp (*68*, *69*) has studied and reviewed the defense mechanisms of gastropods. Various foreign materials were found to elicit different responses when injected into the gastropod, *Australorbis glabratus* (*68*), but none of these responses resembled neoplasia. Labbé (*30*) introduced tar into the mantle of *Doris tuberculata* in an attempt to produce neoplasms, since he thought tar might be carcinogenic to mollusks. He interpreted the response to be cancerous, because the cells were hypertrophied and multinuclear and displayed an "invasive" growth pattern. However, the response in *D. tuberculata* was different from malignancies in mammals. I believe that Labbé did not observe a cancer or even a benign neoplasm but, in reality, a highly cellular wound response. The results of Jullien (*24*) corroborate my evaluation, because he categorically stated that tar was not carcinogenic to cephalopod mollusks, which agrees with the results of Thomas (*66*, *67*) who could not duplicate Labbé's results in other invertebrate animals.

Cephalopoda

Tar was injected into three different cephalopods, *Seiche* sp., *Poulpe* sp., and *Eledone* sp., by Jullien (*24*) with the belief that it was possibly carcinogenic to these mollusks. An oval nodule was always formed at the site of the injection, and the subsequent phenomena were similar except that they proceeded at different speeds, depending on the species of cephalopod used. The nodules, composed entirely of eosinophilic hemocytes and fibroblasts, were similar to the inflammatory reactions produced in cephalopods previously (*22*, *23*) and subsequently (*28*). Jullien's (*24*) experiments showed that tar was not carcinogenic in cephalopods, but acted like any other foreign particle and elicited an inflammatory reaction.

Jacquemain *et al.* (*21*) studied the effects of dibenz[*a*,*h*]anthracene on cuttlefish by inserting several crystals of this compound, enclosed in vaseline, beneath the skin of the visceral sac. They found that, when there was close contact between the crystals and the tissues of the cuttlefish, there was a rapidly expanding circular white tumor that developed to a diameter of several centimeters within 5 days. These lesions lacked chromatophores and an epithelial layer. The few hemocytes in the lesions were necrotic and had pyknotic nuclei. At the center of the lesions there was a thickening of the connective tissue, which appeared to be the main reason for the tumorous appearance. These lesions were unlike anything these authors had observed previously in their experiments on inflammation and wound repair in the cuttlefish, and differed greatly from the wound responses elicited by the insertion of tar (*24*) in cephalopods. There was no cellular reaction oriented in relation to the injurious agent (dibenz[*a*,*h*]anthracene), and the mollusks appeared unable to stop the rapid neoplastic proliferation around the carcinogenic substance (*21*).

The effect of injections of several substances into cephalopods was subsequently studied by Jacquemain and Jullien (*20*). They found that metadichlorobenzene, ortho- and para-chloronitrobenzene, meta- and para-dinitrobenzene, when injected into the visceral sac of cuttlefish, provoked lesions that were analogous to those Jullien found to be caused by tar (*24*). They (*20*) found that ortho- and para-dichlorobenzene, metachloronitrobenzene, orthodinitrobenzene, and peroxide all had a strong influence on the tissue, causing a loss of pigmentation and a lesion unlike an ordinary wound response, but not as proliferative as the tumors previously found after the injection of dibenz[*a,h*]anthracene (*21*).

Jullien *et al.* (*26*) injected crystals of dibenz[*a,h*]anthracene into the visceral sac of the octopus (*Poulpe* sp.) and found lesions 8–10 mm in diameter in 4–5 days, but noted that they were not as proliferative as the lesions found in the cuttlefish (*21*). Histologically, there was an intense inflammatory reaction throughout the area of injury. The lesions produced were not extensive or proliferative and did not appear to be neoplastic.

Jullien *et al.* (*27*) subsequently noted a similar reaction in the cuttlefish. However, the inflammatory response was more pronounced in the cuttlefish (*27*) than in the octopus (*26*), and the cuttlefish was capable of rejecting the carcinogenic substance. Dibenz[*a,h*]anthracene inserted into mollusks was apparently capable of producing either an intense inflammatory response which effectively isolated the irritant (*26*, *27*) or highly cellular tumors that evoked only a weak inflammation that did not restrain the rapidly proliferating neoplastic tissue (*21*).

DISCUSSION

One of the main problems involved in studying invertebrate neoplasia is the difficulty of distinguishing between neoplasia, hyperplasia, and reactions to injuries in the lower animals. Steinhaus (*62*) recently pointed out the need for research on invertebrate neoplasia and noted the lack of trained investigators capable of conducting this research.

Recently, Harker (*14*) and Harshbarger and Taylor (*16*) have given excellent reviews of both the types and causes of tumors in insects. From these reviews, it can be seen that there is a tremendous amount of work being performed on the study of insect neoplasms and related disorders. In comparison, there is only a miniscule amount of interest in other invertebrate groups. The extensive study of neoplasia in insects is probably related to several factors, among which are the genetic knowledge available on insects; the ease of handling and caring for insects; readily available numbers of animals to give statistical support to experiments; and easy examination of the individuals. With the exception of genetic knowledge, all of these features may be found among the mollusks, which are economically one of the most important groups of invertebrates, probably second only to the arthropods.

An understanding of neoplastic processes in the mollusks and in the invertebrates in general is needed and will result only after a large number of tumors has been carefully studied. Systematic surveys of molluscan populations offer a good method of obtaining extensive numbers of naturally occurring lesions in an attempt to classify molluscan tumors and specify criteria for distinguishing among true neoplasia, hyperplasia, and injury responses in these invertebrates.

The diagnosis of neoplasia in mollusks is generally left to the judgment of the investigator making the examination, who at present has no established principles to aid him. Uncontrolled growth with no apparent stimulus is one criterion of neoplasia in vertebrates, and should be considered the same in mollusks. Any swelling or lump should be suspect as a possible neoplasm in these animals, the same as it would be in vertebrates. However, most of us are not as fortunate as Szabó and Szabó (*63*) who were able to observe rapid proliferation in a tumor on a slug over a 3-month period. Instead, we must rely chiefly on histologic evidence to determine uncontrolled growth (neoplasia). This could be observed as simple *expansion* that pushes aside or crowds out the normal cells present, which was observed in an oyster (*43*). However, it should be pointed out that expansion is not specific for neoplasia and may also occur with proliferative granulomas and hyperplasias. *Infiltration*, *invasion*, or *replacement* of normal tissues by atypical cell types or abnormal arrangements of cells would be indicative of neoplasia, as observed in oysters (*47*, *73*). *Mitotic figures* within a suspect growth, such as those observed in oysters (*5*, *7*, *73*) and a mussel (*2*), would be strongly suggestive of neoplasia because mitosis is rarely observed in normal molluscan tissues (*17*). *Metastasis* from a primary lesion would be indicative of a malignant neoplasm. This has never been observed in a mollusk, but, if present, it could be determined by examining serial sections of the animal possessing it.

It is not known whether molluscan tissues, either normal or neoplastic, are capable of undergoing malignant changes. There are no trenchant criteria for malignancy in mollusks, because no positive cases of malignancy have been reported in these animals. Until more cases of molluscan neoplasia are studied, any distinction between malignant and benign tumors will remain as nebulous as the distinction between neoplasia and hyperplasia. However, if it is possible for molluscan tissues to undergo malignant changes, the simplicity of the mollusk offers an excellent system in which to study both benign and malignant neoplasms. Close laboratory observation of neoplasms in different mollusks might shed some light on the distinction between benign and malignant growths in these animals and on the evolution of cancer.

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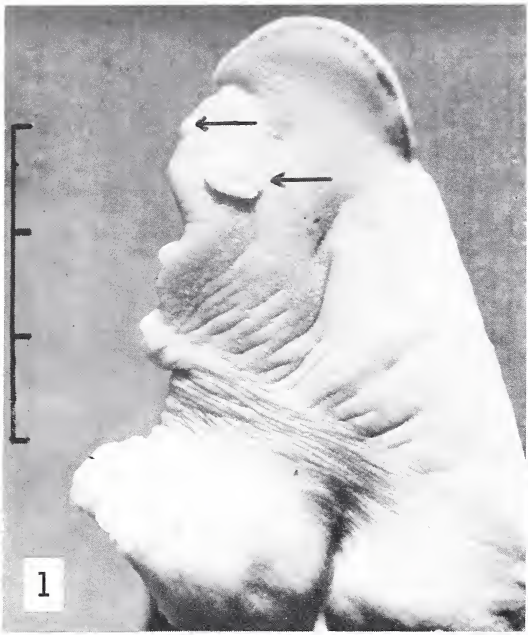
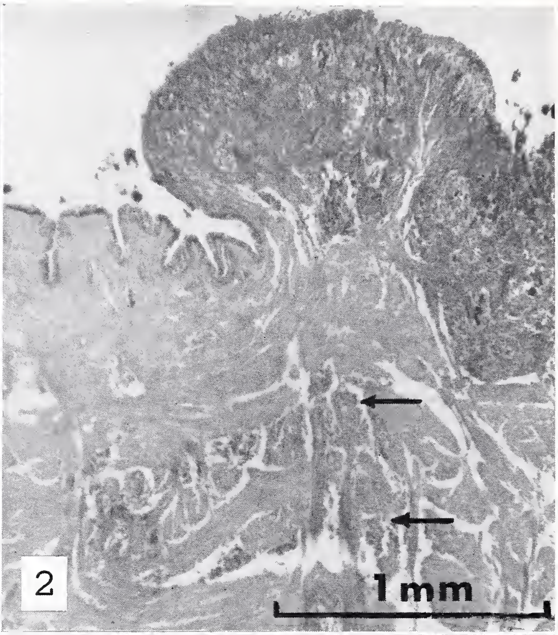
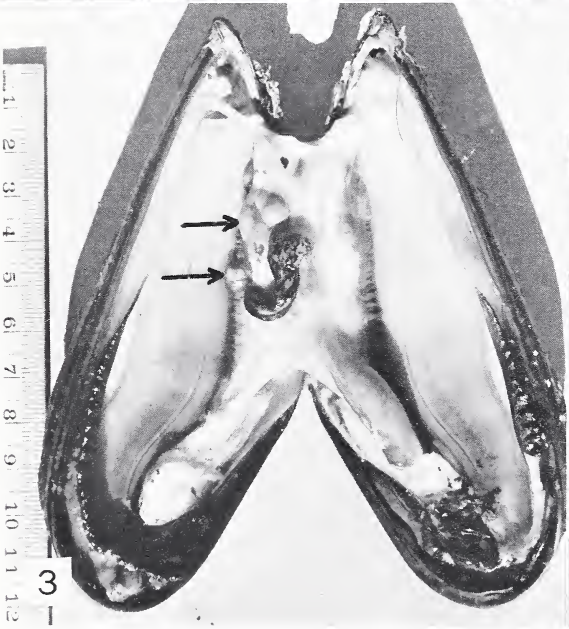






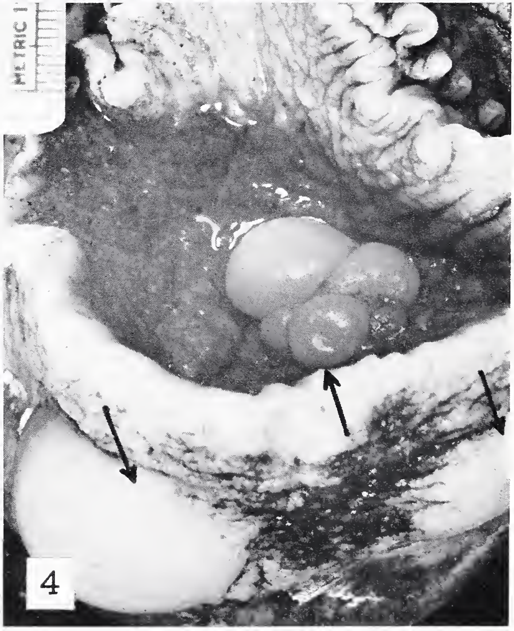
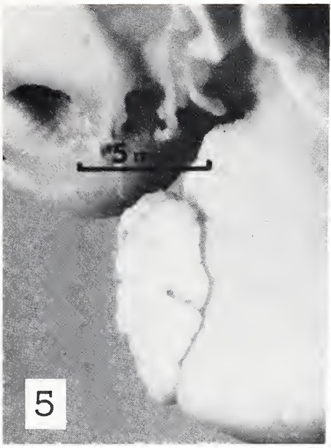


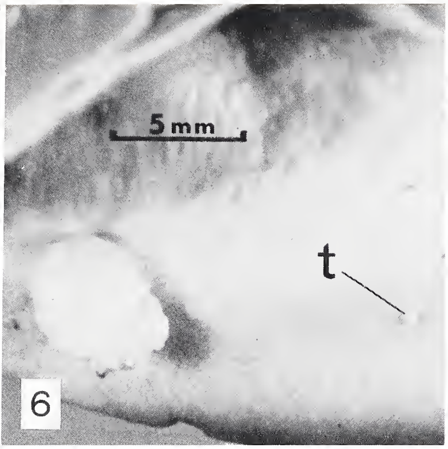
Figure 1.—Foot of gaper clam (*Tresus nuttalli*) with one large polypoid growth (*bottom arrow*) and one small papillary growth (*top arrow*). Scale is in centimeters (*65*).

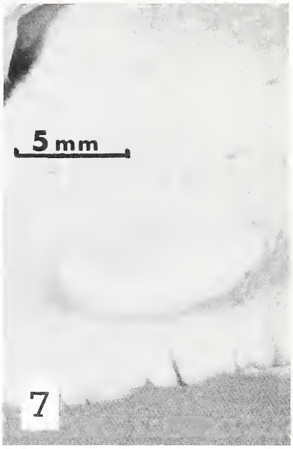
Figure 2.—Microscopical section showing the inflammatory reaction (*arrows*) that occurs deep beneath the surface polyp (*65*). Iron and hematoxylin.

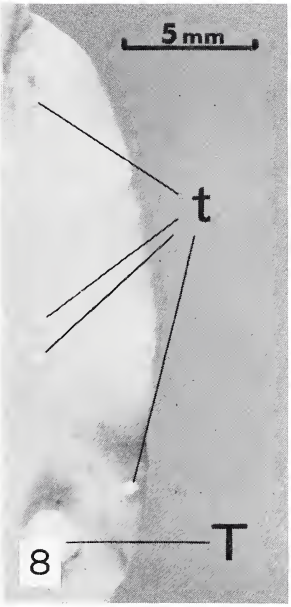
Figure 3.—*Mytilus californianus* with adductor muscle severed and valves opened demonstrating large tumefactions in digestive gland (*upper arrow*) and kidney (*lower arrow*) (*64*).

Figure 4.—Gross view of tumefactions (*arrows*) on the foot of the moon snail (*Polinices lewisii*). *Note* glossy, smooth surfaces lacking pigmentation. Scale is in centimeters (*55*).









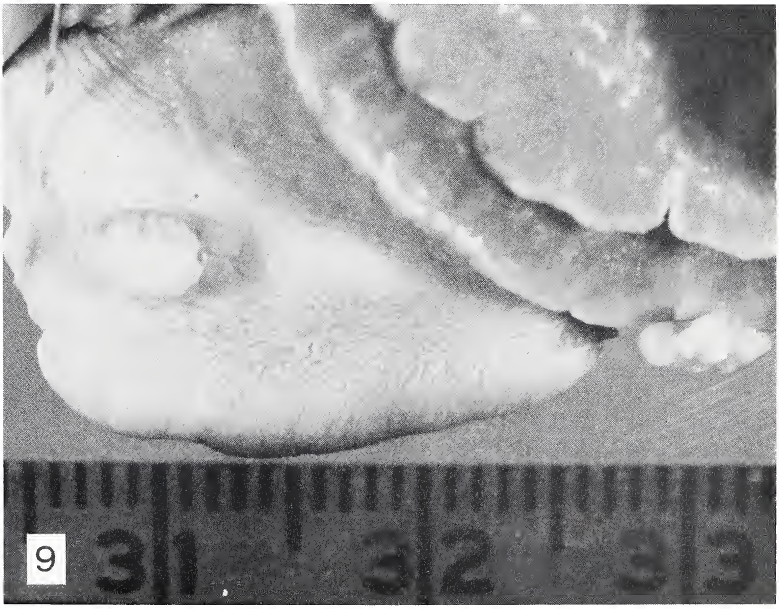


FIGURE 5.—*Anodonta californiensis* with large fungiform tumor on foot.

FIGURE 6.—Freshwater mussel (*A. californiensis*) with large neoplasm and small (t) incipient lesion on foot.

FIGURE 7.—A large bulbous lesion protruding laterally from the foot of freshwater mussel (*A. californiensis*).

FIGURE 8.—Large polypoid tumor (T) and 4 small polypoid growths (t) arising from foot of one *A. californiensis.*

FIGURE 9.—*A. californiensis* with a large polypoid tumor on foot and a similar lesion on one of the gills. Scale is in centimeters.

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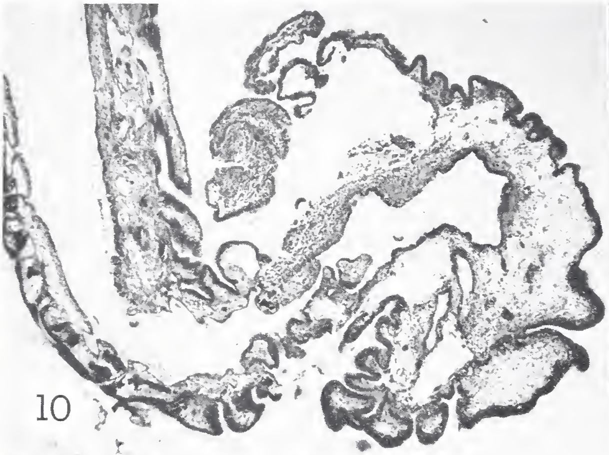
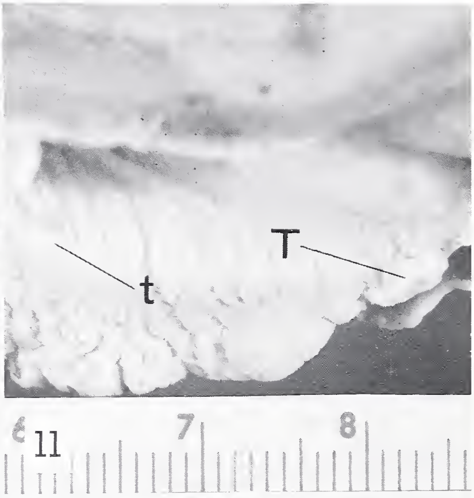
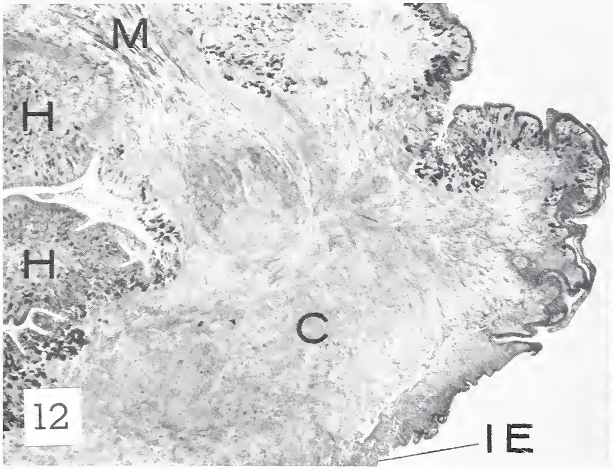






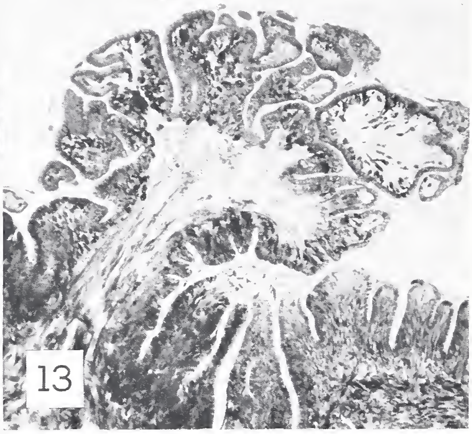
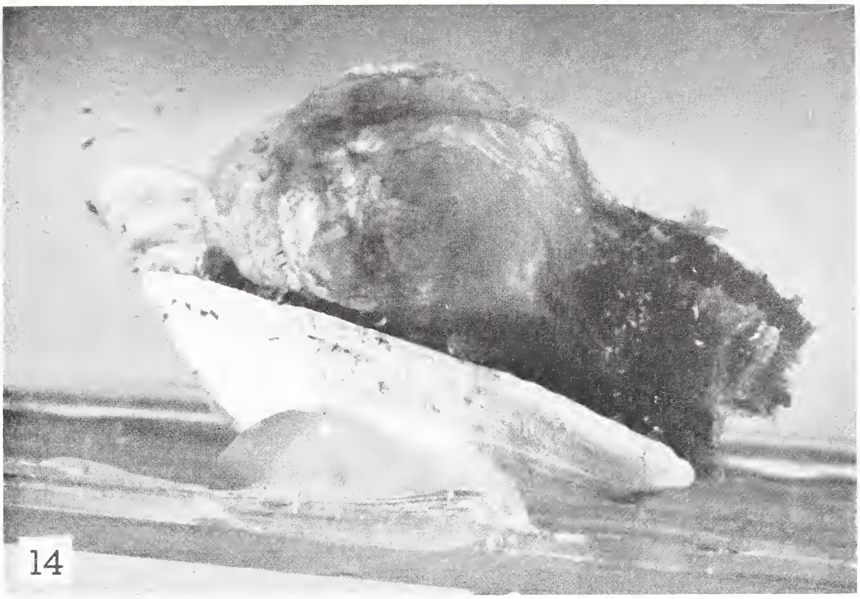


FIGURE 10.—Histologic appearance of gill lesion shown in figure 9. *Note* lack of highly cellular proliferation usually found in true neoplasms. Hematoxylin and eosin. × 45

FIGURE 11.—Freshwater mussel (*Gonidea angulata*) exhibiting large (T) and small (t) lesions on foot.

FIGURE 12.—Miscroscopic appearance of the large lesion on *G. angulata*. *Note* extensive collagenous stroma (C), origin in smooth muscle (M), hyperplastic gland cells (H), and incomplete epithelial covering (IE). Modified Mallory's trichrome. × 45

FIGURE 13.—Histologic appearance of the small growth on *G. angulata*. *Note* highly convoluted epithelial covering and core of smooth muscle. Modified Mallory's trichrome. × 12



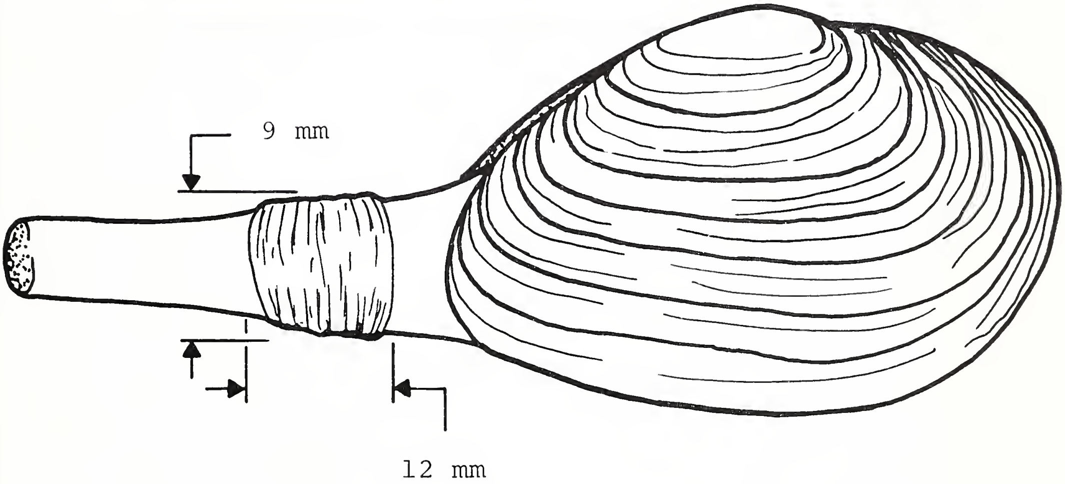


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Figure 14.—Soft-shell clam (*Mya arenaria*) with large tumorous mass protruding from mantle.

Photograph courtesy of Dr. Robert E. Hillman, Battelle Memorial Institute, Duxbury, Massachusetts.

Figure 15.—Sketch of gross appearance of a polypoid tumor on siphons of soft-shell clam (*M. arenaria*) (*41*).

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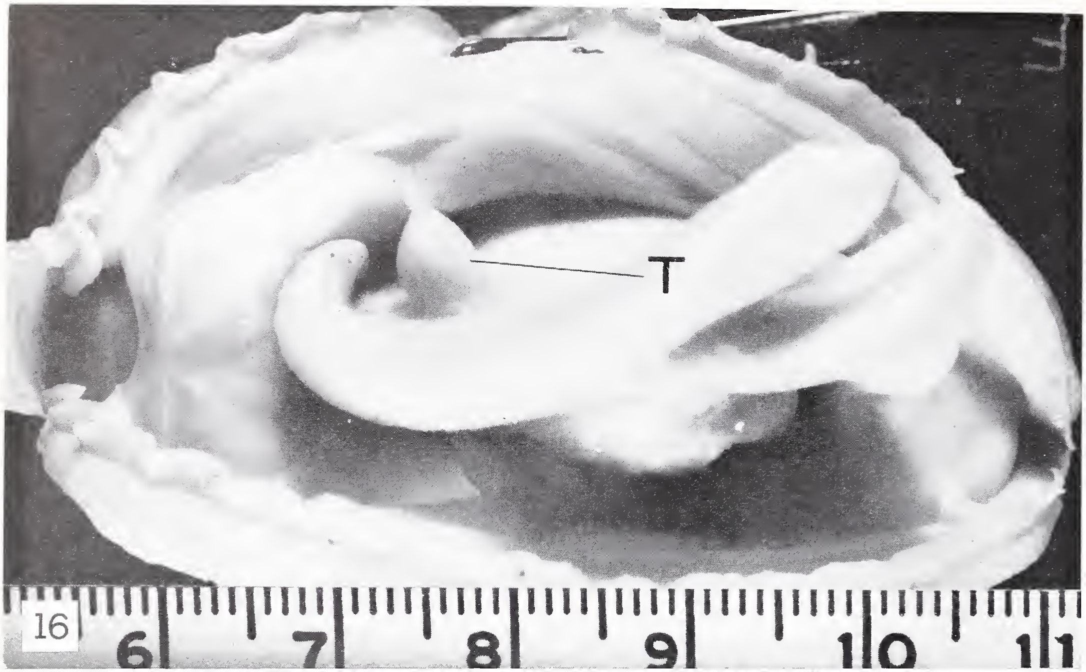


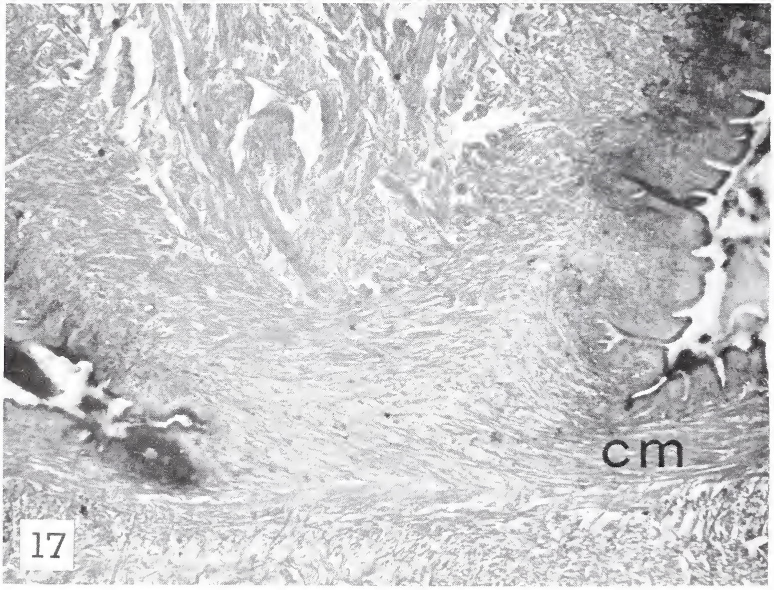
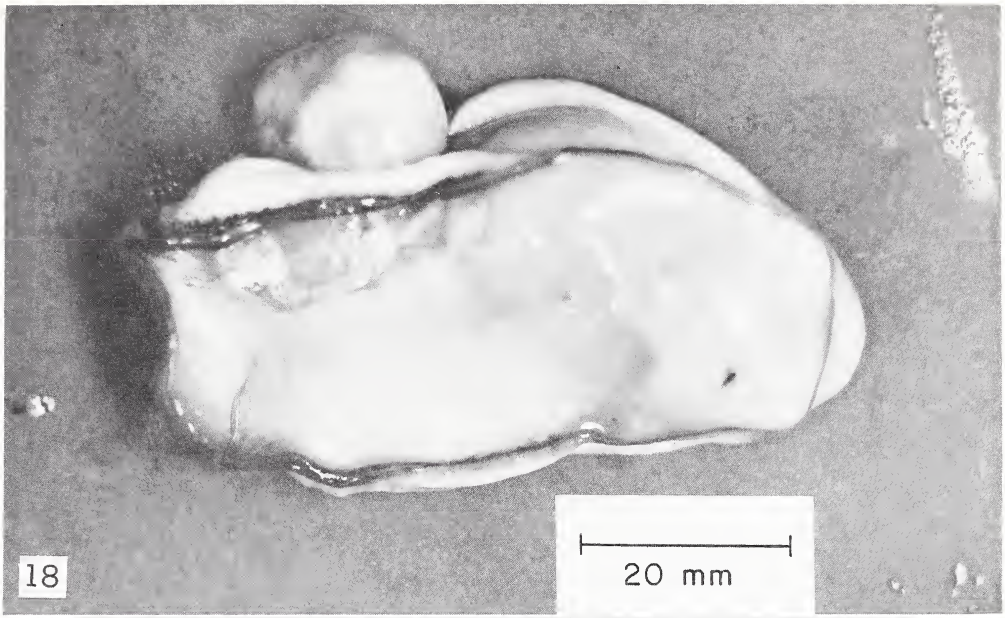


FIGURE 16.—Tumor (T) on foot of butter clam (*Saxidomus giganteus*). Scale is in centimeters.

FIGURE 17.—Histologic appearance of *S. giganteus* lesion. *Note* apparent origin from circular layer of smooth muscle (cm) just beneath epithelium. Hematoxylin and eosin. × 55



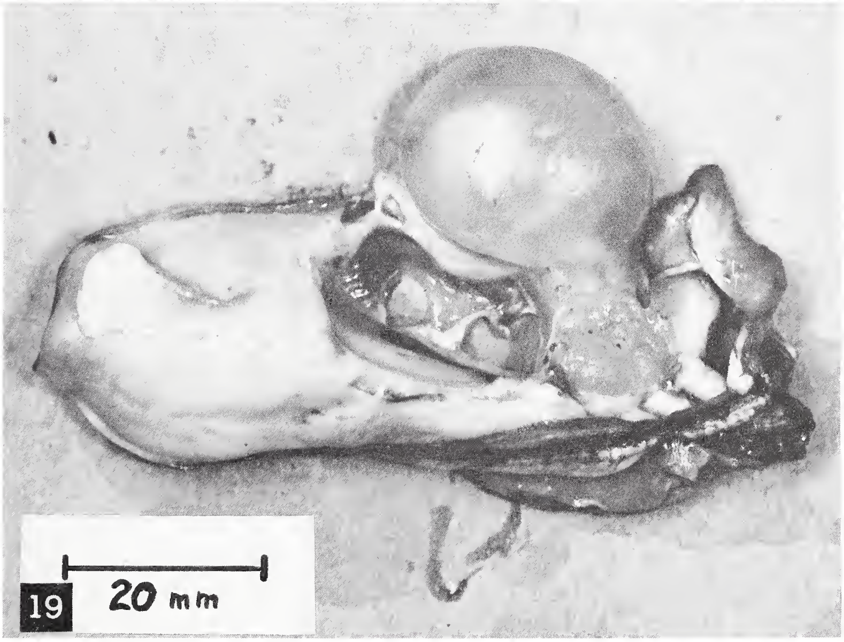


FIGURE 18.—Gross appearance of oyster (*Crassostrea gigas*) with tumor lying dorsal to the rectum (*59*).

FIGURE 19.—Gross appearance of *C. gigas* with tumor-like fecal impaction. *Note* similarity between this growth and the one in figure 18 (*60*).

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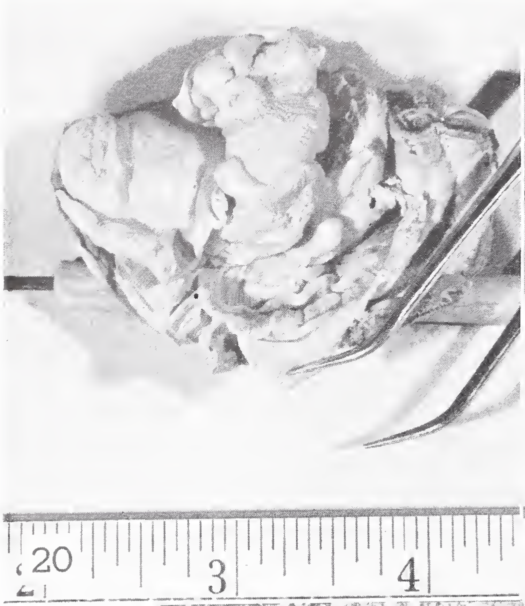

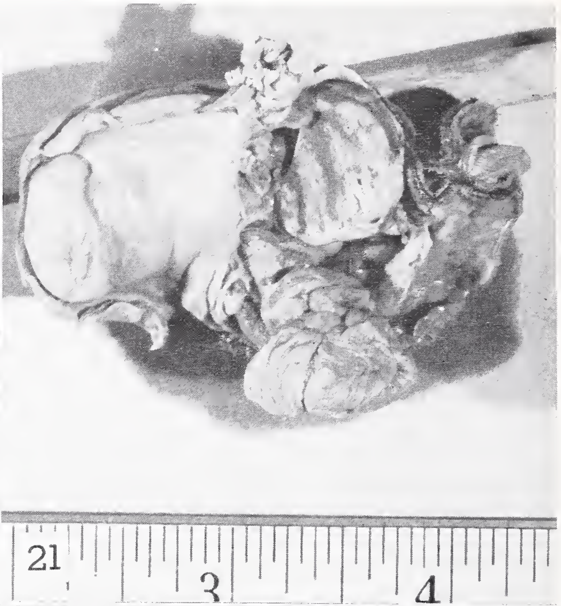

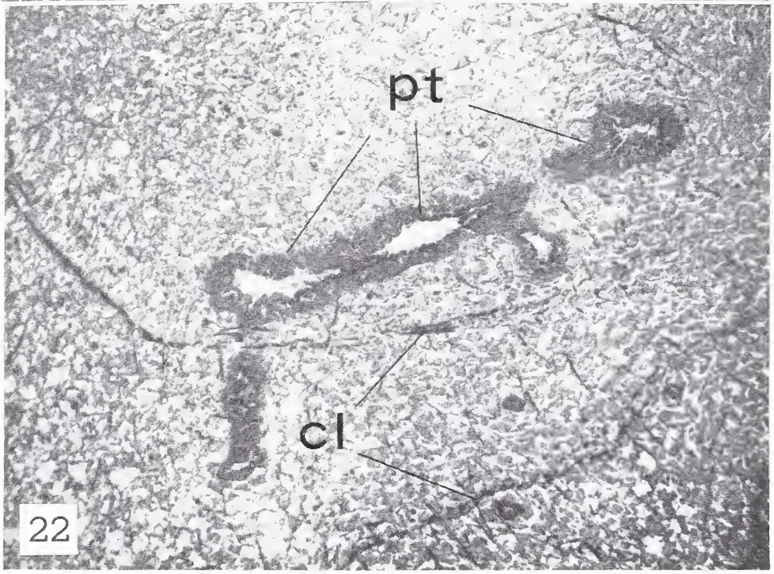

FIGURE 20.—Oyster (*Crassostrea gigas*) with large, crescent-shaped neoplasm arising from suprabranchial chamber. *Note* lesion has perforated the mantle. (Scale is in inches for figs. 20 and 21.)

FIGURE 21.—*C. gigas* with crescent-shaped neoplasm pulled down to show its origin beneath adductor muscle.

FIGURE 22.—Microscopic appearance of *C. gigas* tumor. *Note* "pseudo-tubules" (pt) and concentric layers (cl) of collagen and muscle. Modified Mallory's trichrome. × 140

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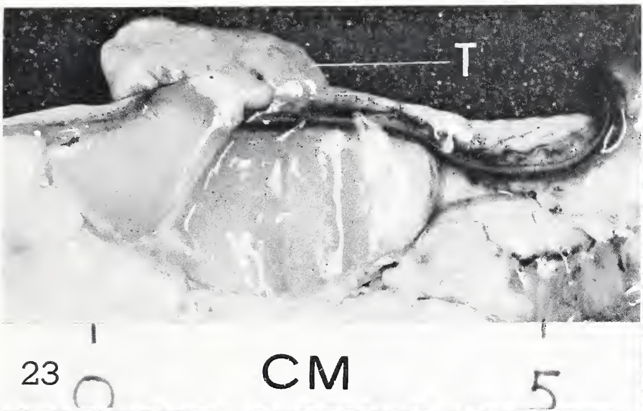

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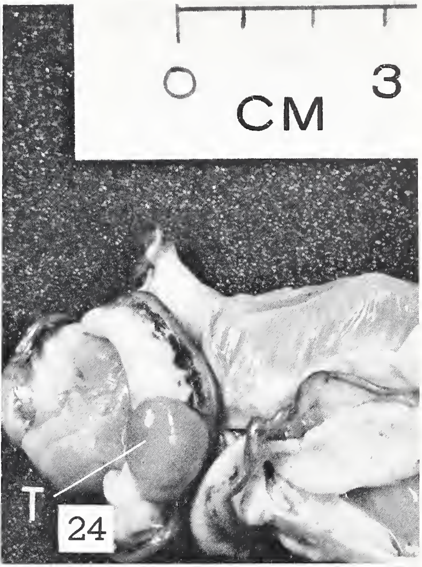

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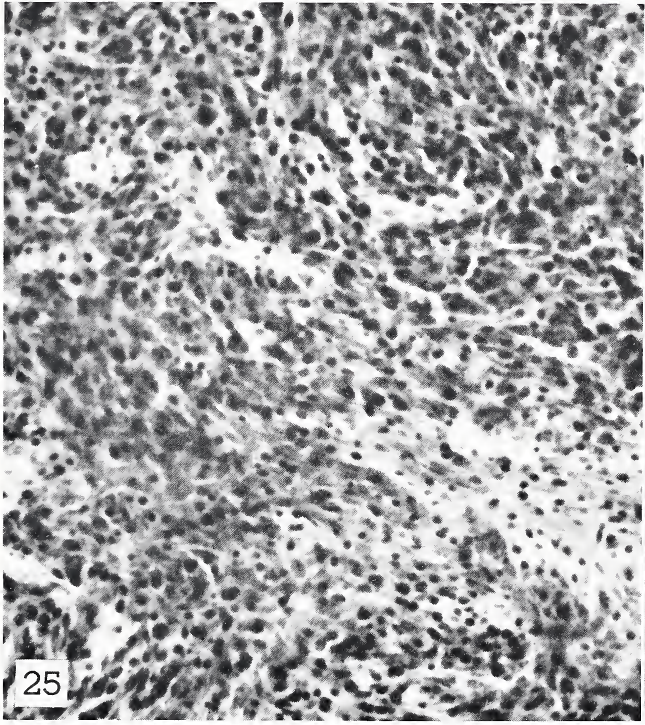

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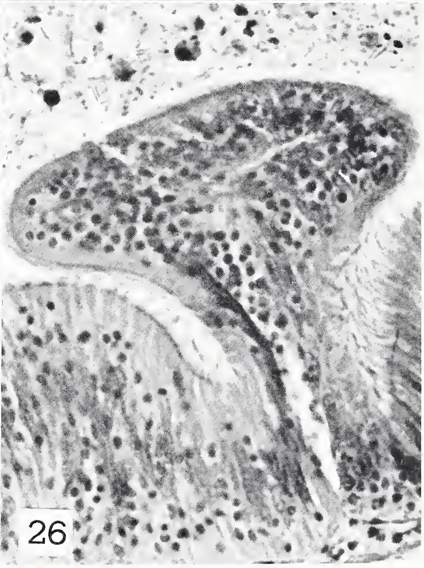

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FIGURE 23.—Oyster (*Crassostrea gigas*) possessing bifurcated adductor muscle with a tumorous mass (T) growing from abnormal anterior portion (*42*).

FIGURE 24.—Dark-green tumor-like lesion (T) arising from inside of oyster's (*C. gigas*) mantle (*42*).

FIGURE 25.—Histologic appearance of internal tumor in oyster (*C. gigas*), showing fibrous connective tissue (*dark*) and collagen (*light*). Modified Mallory's trichrome. × 410

FIGURE 26.—Polypoid growth in intestine of oyster (*Ostrea edulis*). Growth is 0.15 mm high (*29*).

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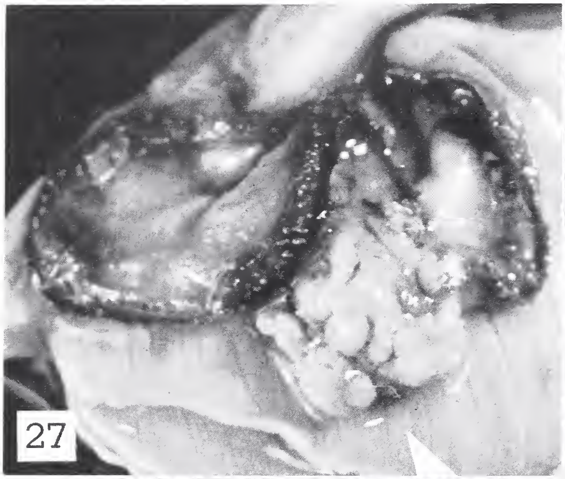

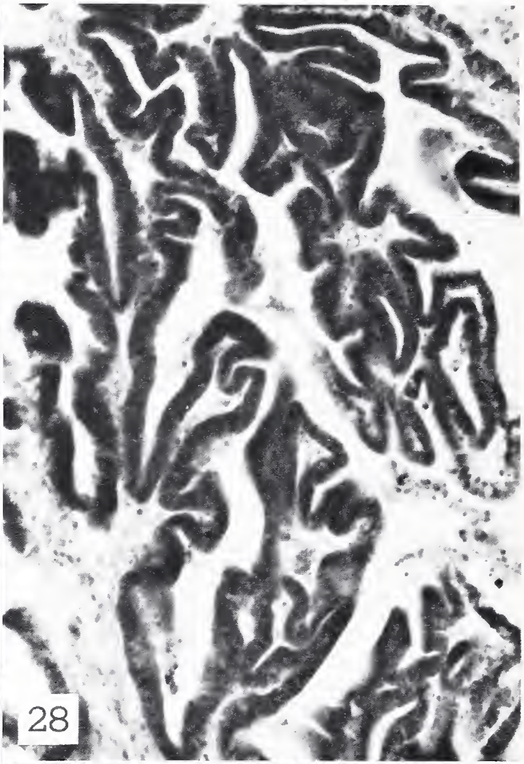

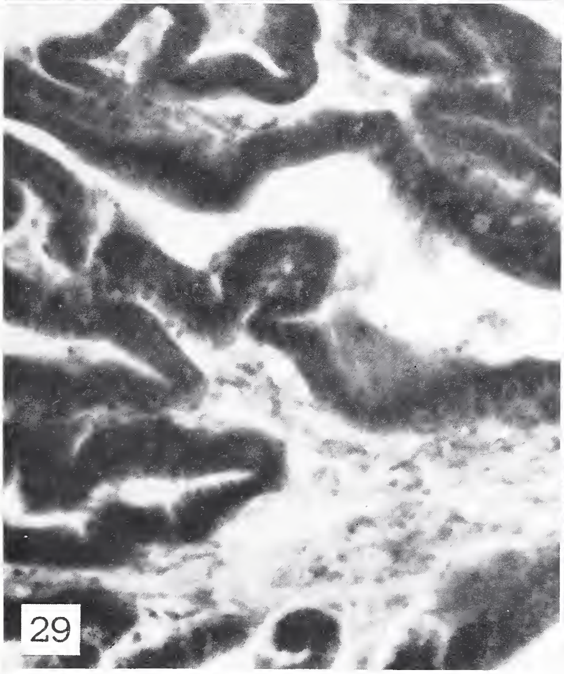

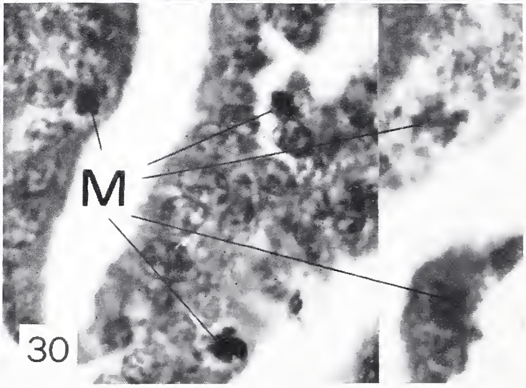

FIGURE 27.—Gross appearance of tumor arising from mantle of oyster (*Crassostrea commercialis*).

FIGURE 28.—Histologic appearance of epithelioma on *C. commercialis*. Heidenhain's iron hematoxylin. × 140

FIGURE 29.—Higher magnification of *C. commercialis* epithelioma. Heidenhain's iron hematoxylin. × 435

FIGURE 30.—High magnification of *C. commercialis* neoplasm, showing numerous mitotic figures (M). Heidenhain's iron hematoxylin. × 910

Photographs courtesy of Mr. Peter Wolf, New South Wales State Fisheries Department, Australia.

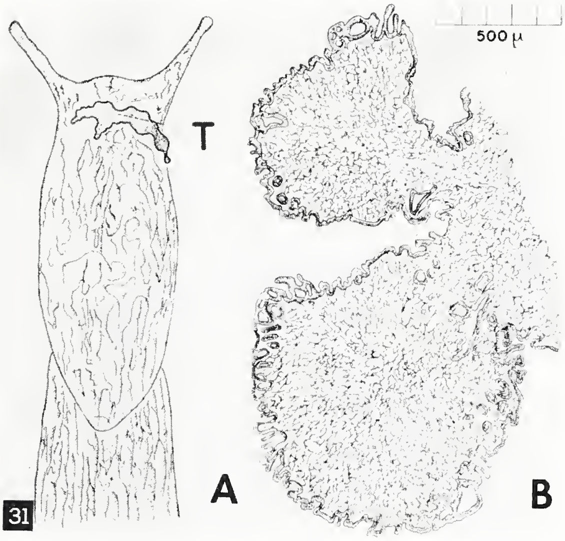

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FIGURE 31.—Gross view (*A*) of slug (*Limax flavus*) showing location of large, spontaneous tumor (T). Cross section (*B*) of two lobes which fell off the tumor shown in (*A*). *Note* atypical arrangement of epithelial cells with connective tissue filling each lobe of tumor (*52, 63*).

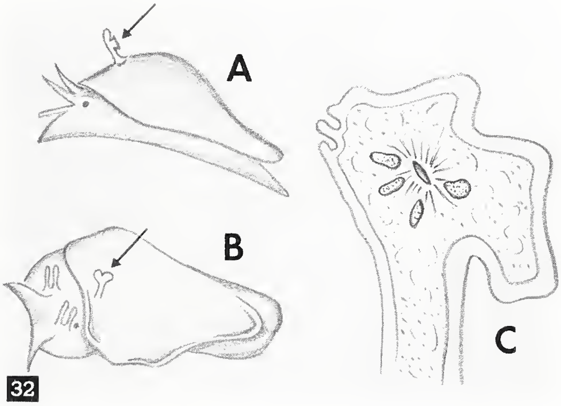

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FIGURE 32.—Fibrous tumor on mantle of snail (*Pleurobranchus plumula*). Gross view (*A* and *B*). Longitudinal cross section (*C*) (*8*).

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Probable Neoplastic Disease of the Hematopoietic System in Oysters, *Crassostrea virginica* and *Crassostrea gigas*

C. AUSTIN FARLEY,[1] *U.S. Bureau of Commercial Fisheries, Biological Laboratory, Oxford, Maryland 21654*

SUMMARY

Six oysters with a proliferative disease, apparently originating in the hematopoietic system, were found during an investigation of pathological, parasitological, and epizootiological factors involved in oyster mortalities over the past 7 years. Approximately 30,000 oysters were examined grossly and histologically. The six diseased oysters exhibited gross and microscopic signs indicative of fatal outcome if the disease had been allowed to run its course. Gross signs were "watery condition" (emaciation), pale digestive gland, and mantle recession. Microscopic signs were invasive infiltration of the vesicular connective tissue by atypical immature hyaline hemocytes, atrophy of gonad, and collagenous fibrosis. Atypical hemocytes were numerous and showed changes consisting of abnormally enlarged nuclei and unusual DNA patterns in pyknotic nuclei. Mitotically dividing hyaline hemocytes were common in the invaded vesicular tissue. Atypical hemocytes in interphase contained abundant RNA in the cytoplasm.—Nat Cancer Inst Monogr 31: 541–555, 1969.

TUMORS HAVE BEEN described from a number of pelecypod mollusks (*1–14*) and extensive reviews have been written concerning the occurrence of tumors in invertebrates (*15–17*). All of the spontaneous neoplasms and "pseudoneoplasms" which have been described in the past were recognized grossly as localized swellings, and were interpreted histologically as noninvasive benign tumors or hyperplastic conditions. Etiology in most cases was unknown; however, oil pollution was suggested as a cause for polypoid growths in soft clams (*6*), and fecal impaction (*8*), parasitic disease (*9*), and injury (*14*) were suggested as causes of hyperplastic responses. Extracts have been isolated from mollusks which apparently inhibit the growth and development of mammalian cancers (*18*, *19*), and, in contrast, mesenchymatous proliferations have been induced in *Sepia* by implanting pellets of 1,2,5,6-dibenzanthracene [2] (*20*).

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[1] I think Dr. Aaron Rosenfield for encouragement and support in the completion of this study; Mrs. Lucretia R. Buchanan and Mrs. L. Cecilia Sullivan for assistance in preparing histologic material; Dr. C. J. Dawe for loan of sections of lymphomas of mice; and Dr. Dawe, Dr. Edward C. H. Schmitt, and Mr. Gilbert B. Pauley for helpful suggestions and discussions during preparation of the manuscript.

Neoplastic disease of the hematopoietic system in mollusks has not been previously reported. This paper reports and describes the occurrence of probable neoplastic disease of the hematopoietic system in oysters. The gross and microscopic pathology, cytochemical studies, and prevalence of this previously unknown condition are presented, and a query is raised as to the origin of hemocytes in oysters. Six cases have been found in two species of oysters—the American oyster, *Crassostrea virginica* (5 cases), and the Pacific oyster, *Crassostrea gigas* (1 case).

Morphologic similarities were remarkable between the undifferentiated germ cells in the gonadal tubules and atypical or normal hemocytes infiltrating other organs and tissues in the neoplastic as well as hyperplastic conditions. Although the simplest interpretation is that the gonadal tubules had been invaded by neoplastic hemocytes, the possibility could not be excluded that the proliferative process arose in primordial germ cells, which then became disseminated to virtually every tissue of the host. A third interpretation has been suggested—that oyster hemocytes may be produced directly from gonadal germ cells and that the disease observed represents neoplastic change within such a line of derivation of hemocytes.

The view that the disease represents a neoplasm of hemocytic origin, analogous to leukemia in vertebrates, is favored. If, however, the cell of origin is the gonadal germ cell, and normal hematopoiesis occurs elsewhere, then the neoplasm should be considered analogous to seminoma or dysgerminoma of vertebrates.

MATERIALS AND METHODS

Oyster samples have been collected since 1960 from the east and west coasts of the United States to study the influence of disease on mortality, with the major emphasis on *Minchinia nelsoni* disease (*21*). Samples of 5–100 oysters were collected during every month of the year. More than 30,000 oysters have been examined histologically.

Histologic Methods

Methods for processing, fixing, sectioning, and staining oyster tissues for pathologic study were described previously (*21–23*) and outlined in detail by Buchanan.[3] Tissues were fixed in Davidson's fluid or Zenker-acetic fluid. In addition to the routine hematoxylin stain (*23*), the following 3 cytochemical procedures, modified for use with oyster tissue, were used on sections of diseased oysters. These techniques have only recently been modified for use with oyster tissue and are still being refined; consequently, the following information is somewhat preliminary.

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[2] *Chemical Abstracts'* nomenclature: dibenz[*ah*] anthracene.

[3] Buchanan, L. R.: Routine Histological Procedures Employed in the Preparation of Shellfish for Pathologic Study. U.S. Bureau of Commercial Fisheries Mimeo: 1–13, 1968. (Unpublished, cited with permission.)

After removal of paraffin from sections by 2 changes of xylene, they were hydrated through descending concentrations of ethyl alcohol to distilled water (all procedures). The digestion procedure for RNA and glycogen [(*24*), chapts. 8 and 14] was inserted at this point and was as follows: Place sections in 0.1% diastace of malt in 0.02 M phosphate buffer of *p*H 6.0 for 1 hour at 45°C. Wash in distilled water and proceed with staining [methyl green-pyronin or periodic acid-Schiff (PAS)].

Feulgen-picro-methyl blue.—Hydrolyze hydrated sections 5 minutes in concentrated hydrochloric acid at room temperature, 30 minutes in 5 N hydrochloric acid at room temperature, or 15 minutes in 1 N hydrochloric acid at 60°C. Wash 2 minutes in running tap water. Dip 6 times in distilled water. Place sections in Schiff's reagent (Lillie's hot method—24, chapt 9) for 15 minutes in refrigerator (5°C.). Dip 6 times in distilled water. Wash 2 minutes in alkaline tap water. Stain 3 minutes in 0.03% methyl blue in saturated picric acid. Dip 6 times in 1 part ethyl alcohol and 9 parts *n*-butyl alcohol. Continue dehydration and differentiation in 2 changes of *n*-butyl alcohol (2 min each). Clear in 2 changes of xylene (5 min each). Mount in Permount. Results: DNA is red, connective tissue is blue, nucleoli and muscle are yellow, cytoplasm various shades of green (figs. 1, 2, 5–19). This technique is a standard Feulgen nucleal reaction, which incorporates a connective tissue stain [(*24*) chapt. 16] as the counterstain. It permits discrimination of DNA from other basic staining elements, and connective tissue from muscle fibers. Critical resolution of DNA patterns, nucleoli, and connective tissue is accomplished with this technique, and it has replaced the more conventional hematoxylin techniques for this study.

Periodic acid-Schiff reaction.—Oxidize hydrated sections 5 minutes in 0.5% periodic acid at room temperature. Wash 5 minutes in running tap water. Dip 6 times in distilled water. Place in refrigerated Schiff's reagent for 10 minutes. Dip 6 times in 0.5% sodium metabisulfite. Alkalize 5 minutes in running tap water. Dip 6 times in distilled water. Mordant 5 minutes in 3% ferric ammonium sulfate. Wash 2 minutes in running tap water. Stain 5 minutes in 10 parts 0.5% aqueous hematin and 1 part dimethyl sulfoxide (*25*) at room temperature. Differentiate in saturated aqueous picric acid until only nuclei remain stained (10–20 min). Wash 2 minutes in running tap water. Dehydrate through ascending concentrations of ethyl alcohol. Clear in 2 changes of xylene (5 min each). Mount in Permount.

Results: PAS-positive substances are red-to-pink and basophilic substances blue-to-black (fig. 3). Glycogen, collagen, reticulin, and mucins are PAS-positive and stain pink-to-red [(*24*) chapt. 7]. The vesicular cells of normal oysters contain heavy deposits of glycogen which are digestible by diastase. Diastase-resistant, PAS-positive substances are found normally in certain secretory cells in the epithelia of the mantle, gills, and digestive tubules, and in basement membranes, pigment cells, fibers of the reticuloendothelial lining of hemolymph vessels, and fine reticuloid fibers of the vesicular connective tissue.

Methyl green-pyronin.—Make up 2% solutions of methyl green and pyronin B. Extract each solution with chloroform in a separatory funnel until the chloroform comes through clear. The lighter aqueous fractions contain the purified stains. Stain sections 6 minutes in a 1:1 mixture of the above aqueous fractions. Blot excess stain with bibulous paper. Dip 6 times in *n*-butyl alcohol. Differentiate 5 minutes in fresh solution of *n*-butyl alcohol. Clear in 2 changes of xylene (5 min each). Mount in Permount.

Results: RNA is red and DNA blue-green (fig. 4). Basic staining substances that stain red with pyronin and are digestible with diastase are tentatively being interpreted as RNA [(*24*) chapt. 8]. The chromatin of nuclei stains blue-to-blue-green. Zenker-fixed sections gave inferior results with this stain. Sections of normal oysters show heavy deposits of RNA in nucleoli, in the cytoplasm of hyaline hemocytes and gametocytes, and in the cytoplasm of cells in the tubules of the digestive diverticula, and the distal gill epithelium.

DESCRIPTION OF DISEASED OYSTERS

Although I have found benign tumors previously during 7 years' study of oyster disease proliferative, invasive neoplasms were seen in only 6 of 30,000 oysters examined. These neoplasms involved the hyaline hemocytes (*21*) of the hemolymph system. Each diseased oyster will be described individually.

Case 1

Species, gross appearance, and physical data—C. virginica: male; length, 5 cm; estimated age 2 years. The condition was watery (emaciated); recession of the mantle and color of digestive gland (*21*) were not recorded; collected from Bachelor's Point Bar, Choptank River, Chesapeake Bay, Maryland, July 18, 1961. Salinity was 8.35‰. Fixation with Zenker-acetic fixative.

Histopathology and cytology—Intensive, diffuse infiltration of the vesicular connective tissue by hyaline hemocytes (fig. 5), apparent hemocytosis, and general atrophy of the gonad were noted. Differentiating gametes were few and indication of collagenous fibrosis was scant.

Hyaline hemocytes average 5 μ in diameter and contained nuclei with an average diameter of 2.9 μ (fig. 6). The cytoplasm of these cells contained much RNA. Nucleoli were prominent in resting nuclei. Mitotic stages (fig. 6) were present wherever invasion had occurred, at a rate of 1 to as many as 8 mitotic figures per oil immersion field. Mitosis seemed to be most frequent in the region of the gonad (fig. 7). Atypical hemocytes were common. Pyknosis resulted in nuclei with solid masses of DNA, crescent-shaped nuclei, and cells in which the chromatin was dispersed throughout the cytoplasm as particles of various sizes and shapes. Enlarged nuclei up to 6 μ in diameter, nuclei with crenulated nuclear membranes, and oval, teardrop-shaped, and indented nuclei were detected.

Case 2

Species, gross appearance, and physical data—C. virginica: female; length, 16.5 cm; an old adult. Collected from Mecox Bay, Long Island, New York, December 22, 1965. Salinity was marine. Fixation with Davidson's fluid. Condition was watery and mantle recession was evident.

Histopathology and cytology—All tissues showed intensive diffuse infiltration by enlarged bizarre-appearing hyaline hemocytes (figs. 1 and 8). Apparent hemocytosis and mild collagenous fibrosis occurred in the vesicular connective tissue exterior to the gonad. Differentiated gametes were rare.

Hyaline hemocytes averaged 6.2 μ in diameter and contained nuclei (4.3 μ) that were more than twice the size of normal hemocytes. The cytoplasm of these cells had sparse amounts of RNA. Mitotic figures were infrequent in blood spaces (fig. 9). Most cells in the primordial epithelium of the gonad were dividing by mitosis (fig. 10) and did not appear to be forming gametes. Nuclei of enlarged hyaline hemocytes (often with clear regions on one end) were usually elongated and contained a prominent nucleolus (fig. 9).

Case 3

Species and physical data—C. gigas (female). Gross appearance was not recorded. Collected from Matsushima Bay, Japan, August 29, 1966. Salinity was marine. Fixation with Davidson's fluid.

Histopathology and cytology—Diffuse infiltration by mixture of hemocyte types was obvious throughout tissues (fig. 11). Resorption of gametes was apparent.

Hyaline hemocytes contained much RNA. Mitosis in these cells occurred in connective tissue and hemolymph sinuses (fig. 12). As many as 8 figures per oil immersion field were seen but usually the number averaged less than 1. Pyknotic nuclei, cells with DNA inclusions in cytoplasm, and binucleate hemocytes were observed. Mitosis also was in process in the primordial epithelium of the gonad (fig. 13).

Case 4

Species, gross appearance, and physical data—C. virginica: age, 1.5 years; sex, indeterminate. Condition was watery with pale digestive gland and signs of mantle recession. Sample was from 1966 St. Mary's River set, introduced onto Marumsco Bar, Pocomoke Sound, Maryland, in May 1967, and collected September 25, 1967. Salinity was 15.15‰. Fixation with Davidson's fluid.

Histopathology and cytology—Evident features were heavy invasion of the connective tissue by hyaline hemocytes (fig. 14), apparent hemocytosis, general atrophy of tissues, light collagenous fibrosis, and several large lesions encapsulated by thin layers of collagenous tissue.

Approximately 40% of the hyaline hemocytes appeared viable and showed large amounts of RNA in the cytoplasm. These cells averaged 5 μ in diameter and had nuclei which averaged 3.6 μ (fig. 15). Approximately 60% of the hyaline hemocytes were atrophied and pyknotic, had little RNA, and averaged 3 μ in diameter; nuclei averaged 2 μ. Mitotic figures per oil immersion field were as many as 5. Atypical forms resembled those described for Case 1.

Case 5

Species, gross appearance, and physical data—C. virginica: male; age, 1.5 years. Condition was watery, digestive gland was pale, and there were signs of mantle recession. Specimen was collected November 16, 1967, from the same population as oyster in Case 4. Salinity was 16.67‰. Fixation with Davidson's fluid.

Histopathology and cytology—There was heavy infiltration of the connective tissue by hyaline hemocytes (fig. 16), apparent hemocytosis, and general atrophy of gonadal tissue. Dense infiltrations of hyaline hemocytes separated the basement membrane from the intestinal epithelium, and collagenous fibrosis was heavy in the region of the gonadal alveoli and in the connective tissue of the mantle (fig. 3).

Approximately 70% of the hyaline hemocytes (5.2 μ, diameter) contained RNA in the cytoplasm and enlarged nuclei (3.7 μ) (fig. 2). Mitosis in hemocytes was common throughout the tissue (fig. 17). Division figures per oil immersion field were, as many as 10, and 3–5 per field were not unusual. At least 1 figure could be found in almost every field. Tissues contained bizarre forms of hemocytes similar to those described in Case 1.

Case 6

Species, gross appearance, and physical data—C. virginica: Length, 6 cm; age, 2 years; sex, indeterminate. Condition was medium (*21*), and the digestive gland was pale. This oyster was taken from an observation tray of 1965 St. Mary's River set which was introduced onto Marumsco Bar, Pocomoke Sound, Maryland, in May 1966, and then into the Wicomico River at Whitehaven, Maryland, in September 1966; date of the collection sample was December 18, 1967. Salinity was 4.5‰. Fixation with Davidson's fluid.

Histopathology and cytology—Connective tissue was infiltrated diffusely by hyaline hemocytes (fig. 18), and gonadal tissues were atrophied. Collagenous fibrosis was moderate in gonad regions and in connective tissue adjacent to the mantle epithelium.

Hyaline hemocytes averaged 4.2 μ in diameter and contained nuclei which averaged 3.3 μ. The cytoplasm of these cells was rich in RNA. Mitotic figures were abundant in hemocytes in all lesions (fig. 19). Oil immersion fields contained on the average more than 1 division figure and several fields had as many as 10. Bizarre forms were common, but enlarged nuclei were rare.

PATHOLOGIC IMPLICATIONS

Pathologic Manifestations of Proliferative Hemolymph Disease

The gross signs and histopathology of this disease in the oyster have been listed in table 1, which compares characteristics of the 6 cases with characteristics found in normal oysters, and with characteristics of oysters infected with *M. nelsoni* (*21*).

Poor condition (emaciation), pale digestive gland, and mantle recession are nonspecific signs of serious pathologic diseases which usually result in death of the affected oysters (*21*). Atrophy in the gonad is believed to be caused by tissue damage, and fibrosis is an attempt to repair it. Histologically, increased numbers of infiltrating hyaline hemocytes are usually accompanied by phagocytopenia (*21*). These manifestations are all characteristic of lethal disease, of which *M. nelsoni* disease is an example. Despite the apparent massive increase in hemocyte numbers in known diseases, mitotic figures in the hemolymph are rare. Increased concentrations of RNA in the cytoplasm of hyaline hemocytes suggest that these cells are immature and actively participating in a proliferative process. Pyknosis of hyaline hemocyte nuclei is normally seen only in moribund oysters and lacks the bizarre appearance described for the various cases. Mitosis in the primordial epithelium of the gonad is a normal, seasonal occurrence, but the occurrence of mitosis of cells in the hemolymph is extremely rare.

All of the cases had one or more gross signs of disease and the neoplasms all appeared to have originated in the vicinity of the gonad. All had hyaline hemocytosis, infiltration, and mitosis in the hemolymph. Most apparently had hemocytopenia (*21*) of phagocytes and pyknosis of hyaline hemocyte nuclei. In three cases hemocyte nuclei were enlarged.

Gross signs indicated that Cases 2 and 6 were the least advanced and 4 and 5 the most advanced. Cases 1 and 6 closely resembled each other cytologically; both had normal-sized nuclei and generally diffuse dissemination. Cases 4 and 5 were similar cytologically; each had enlarged nuclei and diffuse infiltration by proliferating hemocytes. Cases 2 and 3 had special characteristics: Case 2 showed greatly enlarged, atypical nuclei with little evidence of active proliferation in the hemolymph, and case 3 a mixture of cell types with no enlargement of nuclei.

Table 1.—Comparison of pathologic manifestations of proliferative hemolymph disease in the oyster*

Manifestation	Normal oyster	*Minchinia nelsoni* disease. Advanced infection	Hematopoietic neoplasm in oysters (cases)						Mammalian leukemia	
			1	2	3	4	5	6		
Watery condition	—	+	+	+	?	+	+	—	+	Emaciation
Pale digestive gland	—	+	?	—	?	+	+	+	+	Pale organs
Mantle recession	—	+	?	+	?	+	+	—	—	—
Atrophy of gonad	—	+	+	—	—	+	+	+	—	—
Fibrosis	—	P	—	+	—	+	+	+	P	Fibrosarcoma
Phagocytopenia	—	+	+	—	—	+	+	+	+	Anemia
Hyaline hemocytosis	—	+	+	+	+	+	+	+	+	Leukocytosis
Hyaline hemocyte infiltration	—	+	+	+	+	+	+	+	+	Lymphocytic infiltration
Enlarged nuclei in hyaline hemocytes	—	—	—	+	—	+	+	—	+	Enlarged nuclei in neoplastic cells
Supranormal cytoplasmic RNA in hyaline hemocytes	+	P	+	—	+	+	+	+	+	RNA in proliferative cells
Atypical cells and pyknosis	—	P	+	+	+	+	+	+	+	Atypical cytology
Mitosis (primordial epithelium of gonad)	P	P	+	+	+	—	P	P	+	Dysgerminoma
Mitosis (hemolymph)	R	P	+	U	+	+	+	R	P	Leukemia

*+ = Positive; — = negative; P = possible; U = uncommon (less than 1 per 200 × field); R = rare (less than 1 per section).

The clinical and microscopic manifestations often associated with hematopoietic neoplasms in mouse (fig. 20) (*26*), and man (*27*), which seem to be comparable are: emaciation, cytopenia of various types of non-neoplastic blood cells, leukemic or lymphomatous cell infiltrations, abnormally high cytoplasmic RNA in neoplastic cells, atypical and pyknotic forms, nuclear enlargement and diffuse dissemination, and uncontrolled proliferation. Cases 1, 4, 5, and 6 appear to have the characteristics of hemocytic neoplasms and are interpreted as probable acute cases of spontaneous neoplastic disease.

Case 2 displayed the most bizarre modification of hemocytes and the effects of massive hemocytic proliferation. The paucity of mitotic figures in the invaded tissues, decreased RNA in the cytoplasm of neoplastic cells, and the rarity of cell necrosis are suggestive of a chronic proliferative disorder that had not reached its terminal phase. The relatively large size of nuclei, which should reflect a high degree of immaturity, was inconsistent with other characteristics in this particular case. The occurrence of diminutive nuclei in cases 4, 5, and 6 reflects extensive maturation leading to pyknosis and necrosis. These changes probably signify that the animals were near death.

The interpretation of Case 3 is the most questionable because comparative information on species differences is not available, and because data on gross characteristics were lacking. Further, the atrophy, fibrosis, and phagocytopenia seen in other cases were lacking. It has been included because of the occurrence of atypical hemocytes, pyknosis, and abnormal mitotic activity in hemolymph.

In the 6 cases described, the morphologic similarity of the neoplastic cells to oyster hyaline hemocytes (*21*) suggests that they are of hematopoietic origin. Little is known about the origin of normal hemocytes in oysters (*28*), or mollusks in general (*29*). Feng (*30*), writing of oyster leucocytes, stated that "Literally thousands of oyster histologic preparations have been scanned by the writer and his former colleagues at Rutgers University, but mitotic figures of leukocytes are rarely seen in these cells. . . . Yet we are still ignorant about the origin and fate of these cells." The vesicular connective tissue, reticuloendothelial lining of hemolymph vessels (Pauley, personal communication, 1968), and the kidney epithelium have been suggested as sites of hematopoiesis but definitive evidence is lacking for all of these sites.

In the material that I have examined, mitosis is either totally lacking, or rare in all of these sites. Five of the six cases described here exhibited mitotic activity in the primordial epithelium of the gonad with little or no apparent gametic differentiation. The undifferentiated cells of the primordium resembled hyaline hemocytes morphologically and cytochemically but usually had larger nuclei. The mesodermal origin (the germ layer which is known to develop into hematopoietic tissue in vertebrates) of this primordial epithelium and its location adjacent to large hemolymph sinuses are features which might indicate this as a logical site for normal hematopoiesis. This site may have been overlooked previously because of the expected normal mitotic activity that accompanies the formation of gametes. The undifferentiated primordial layer of the gonad possibly serves 2 purposes: 1) the production of gametes, and 2) the formation of hemoblasts which migrate into hemolymph sinuses and then differentiate into specialized hemocytes, as reported previously for gastropods (*29*). A fibrous tumor composed of cells resembling the primordial cells of the gonad (which was suggested as the primary site of origin) was recently described (*14*). The cell types shown in photomicrographs of this tumor appeared to me somewhat similar to those in the cases that I have presented; the tumor was described, however, as "noninvasive and benign in nature" (*14*). It appears that the 6 cases of proliferative hemolymph disease may have originated in the primordial epithelium of the gonad, and could possibly be similar to dysgerminoma or seminoma; if, however, the gonadal epithelium is actually the site of normal hematopoiesis, then these cases might be considered to be analogous to mammalian leukemia.

Etiology

The cause or causes of hematopoietic neoplasms in oysters are unknown. Conceivably, this condition could be a virus-induced disease; the extremely low incidence, however, suggests a low infectivity rate or a strong innate resistance, possibly created by the presence of a substance such as mercenene (*18*) in most oysters, or by a rarity of transmissible stages of the presumptive virus. It is also possible that carcinogenic substances occurring as pollutants in estuaries might be etiologic agents, but most of the specimens were collected from the least polluted regions of the estuarial systems involved.

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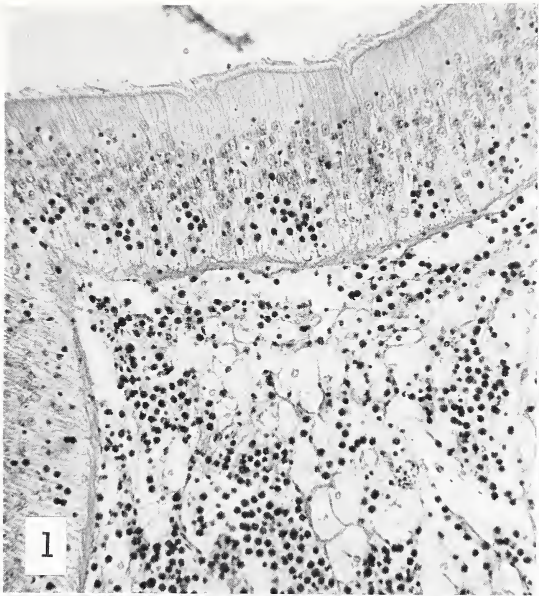
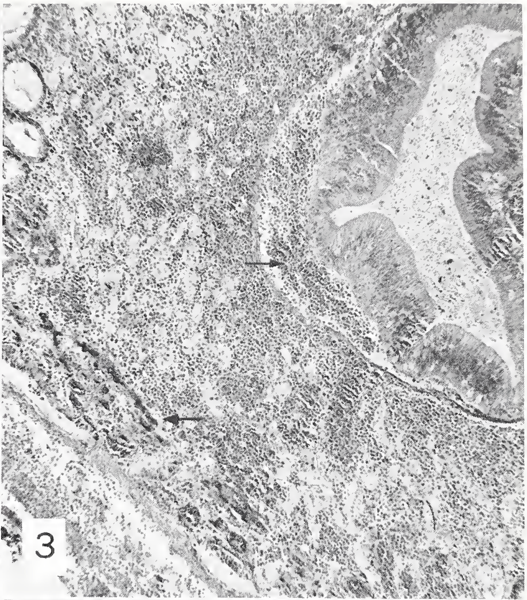
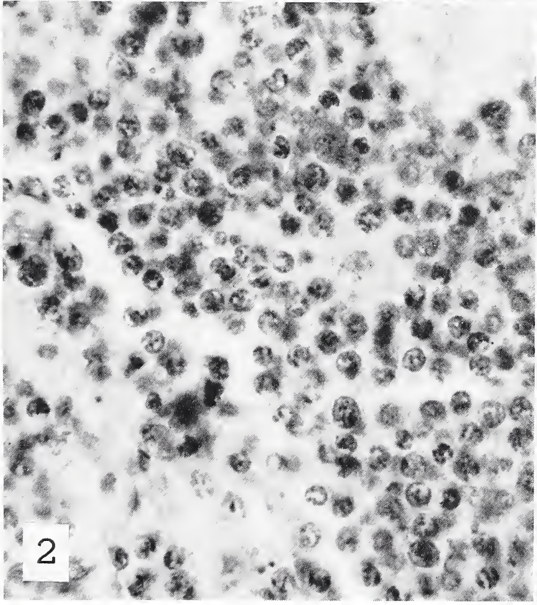






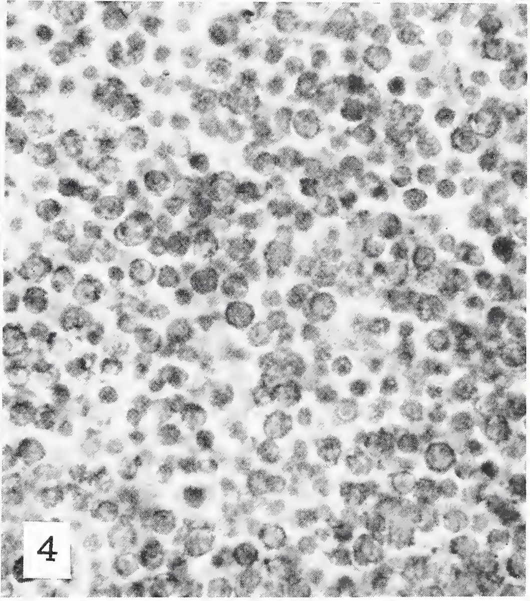
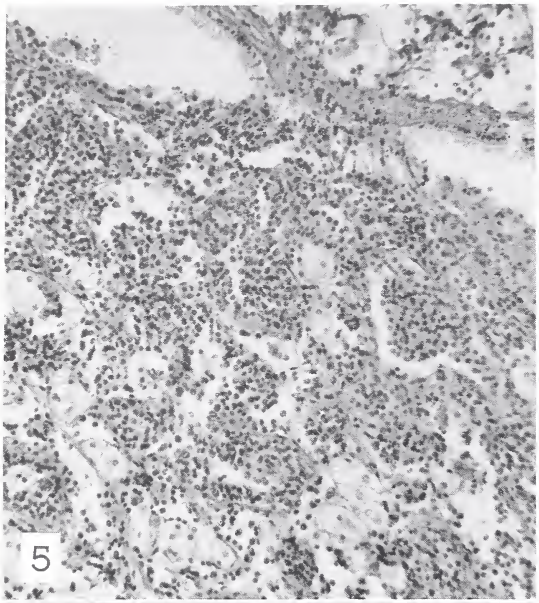


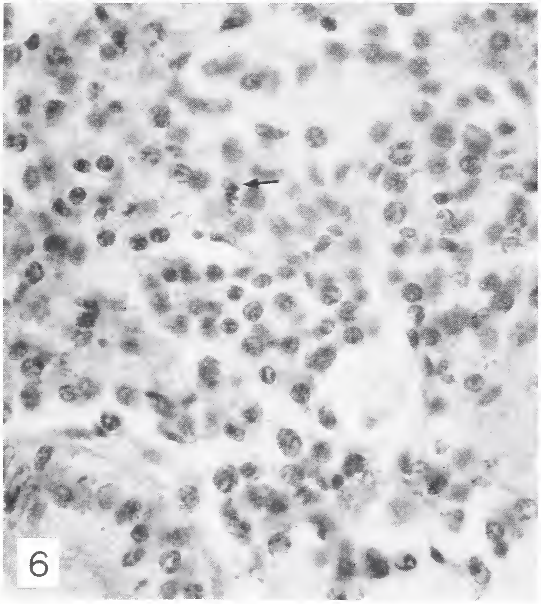
Figure 1.—Gut and adjacent connective tissue, Case 2. *Note* larger densely staining nuclei of neoplastic hemocytes which have invaded connective tissue and penetrated epithelium. Feulgen-picro-methyl blue. × 225

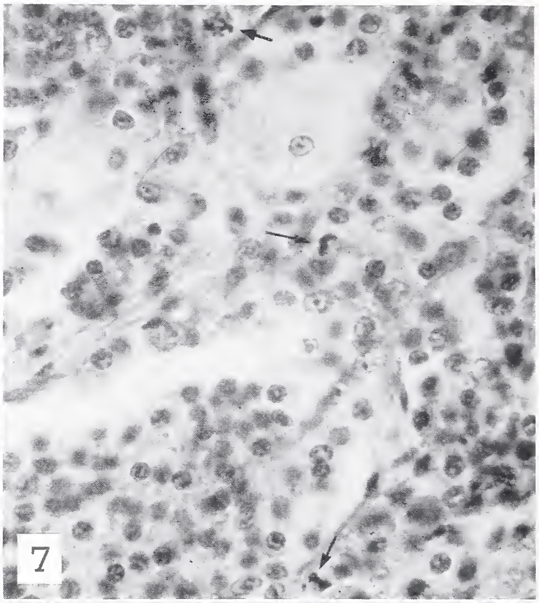
Figure 2.—Neoplastic invasion of connective tissue, Case 5. *Note* nuclei and mitotic figure. Feulgen-picro-methyl blue. × 900

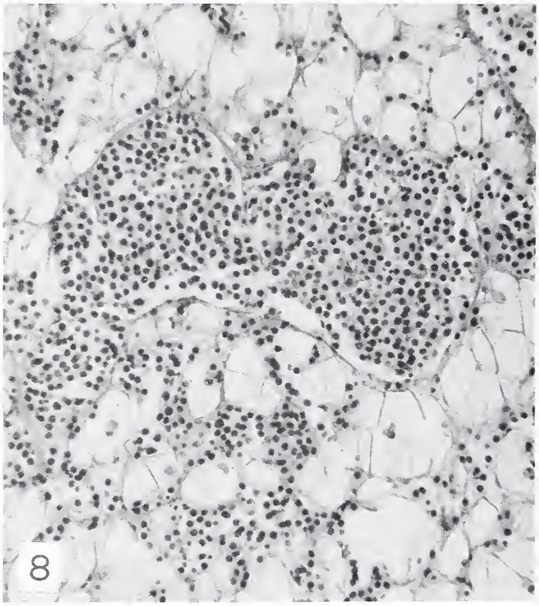
Figure 3.—Neoplastic invasion, Case 5. *Note* distension and separation of gut epithelium caused by dense infiltration of neoplastic hemocytes. *Arrows* show invasion under PAS-positive basement membrane, and PAS-positive fibrosis of gonad. Diastase-digested periodic acid-Schiff (PAS)-dimethyl sulfoxide iron hematoxylin. × 90

Figure 4.—Neoplastic cells, Case 4. *Note* darkly staining RNA deposits in cytoplasm of hemocytes with enlarged nuclei and lack of RNA in hemocytes with smaller nuclei. Nucleoli normally contain RNA. Methyl green-pyronin stain. × 900









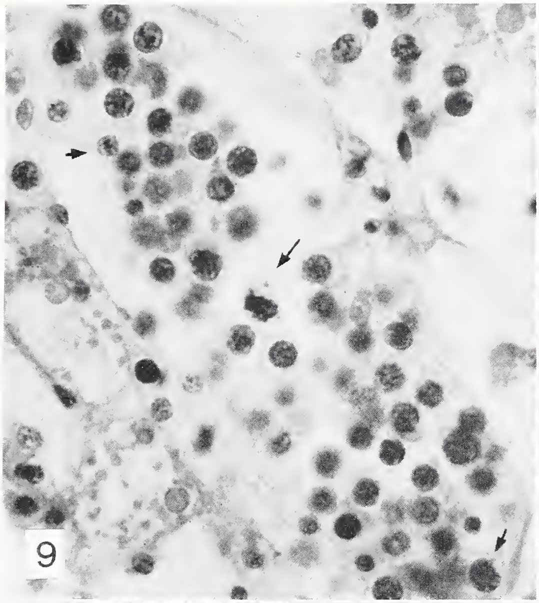
Feulgen-picro-methyl blue stain

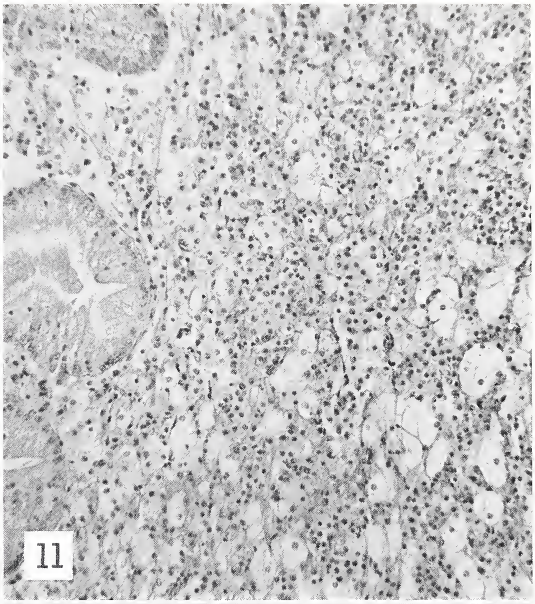
Figure 5.—Case 1: Neoplastic invasion of connective tissue. *Note* extensive involvement of gonadal alveoli. × 225

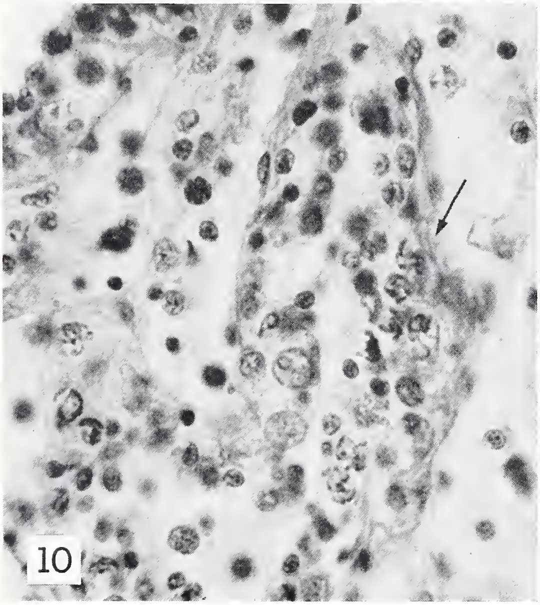
Figure 6.—Case 1: Proliferative area near digestive diverticulum. *Note* mitosis (*arrow*) and variations in size and shape of nuclei. × 900

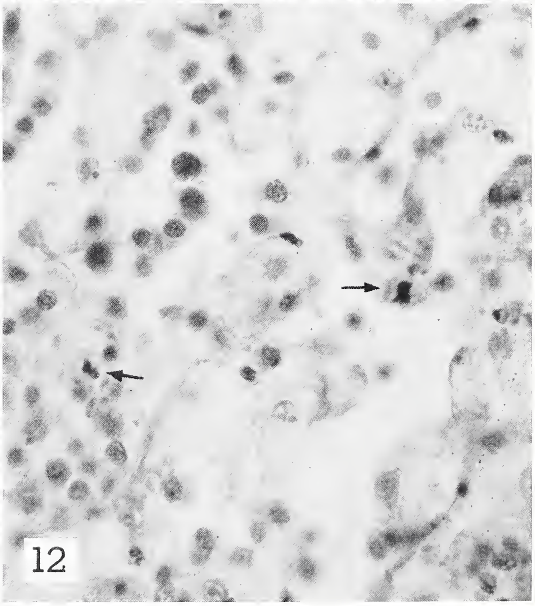
Figure 7.—Case 1: Mitosis (*arrows*) in gonadal alveoli. × 900

Figure 8.—Case 2: Hemolymph sinus containing neoplastic and normal hemocytes. Neoplastic cells, recognized by enlarged densely staining nuclei, can be seen invading connective tissue. × 225









Feulgen-picro-methyl blue stain

FIGURE 9.—Case 2: Hemolymph sinus showing neoplastic cells. *Arrows* show mitotic figure, lytic regions in nuclei, and normal hemocyte. × 900

FIGURE 10.—Case 2: Gonadal alveolus showing mitosis (*arrow*) in primordial epithelium. × 900

FIGURE 11.—Case 3: Infiltration of connective tissue by mixture of hemocyte types. × 225

FIGURE 12.—Case 3: Invasion of connective tissue by hyaline hemocytes. *Arrows* indicate mitotic figures. × 900

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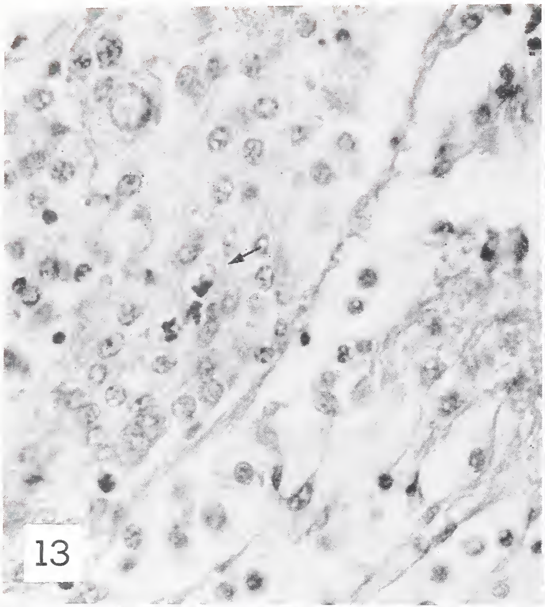
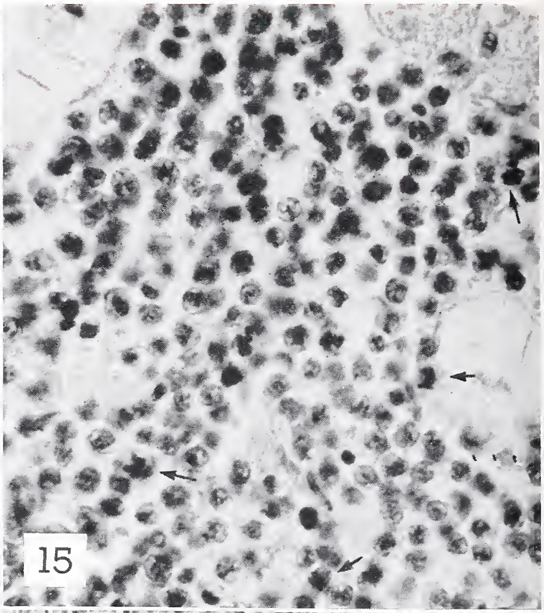
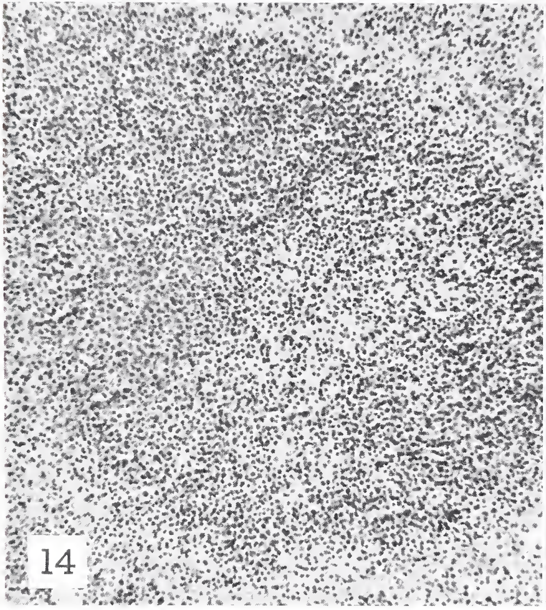
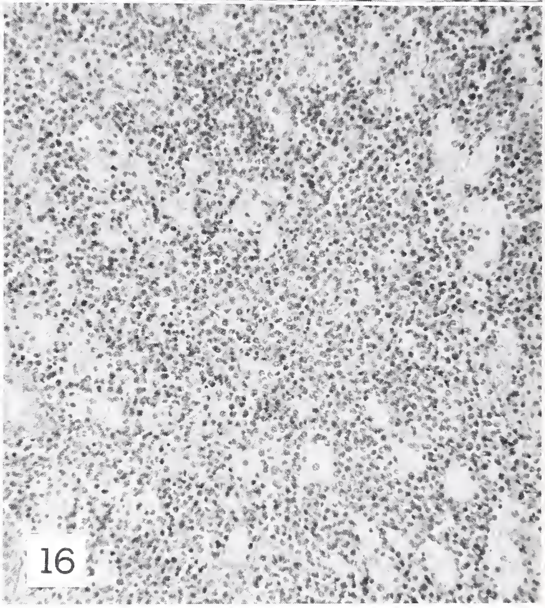








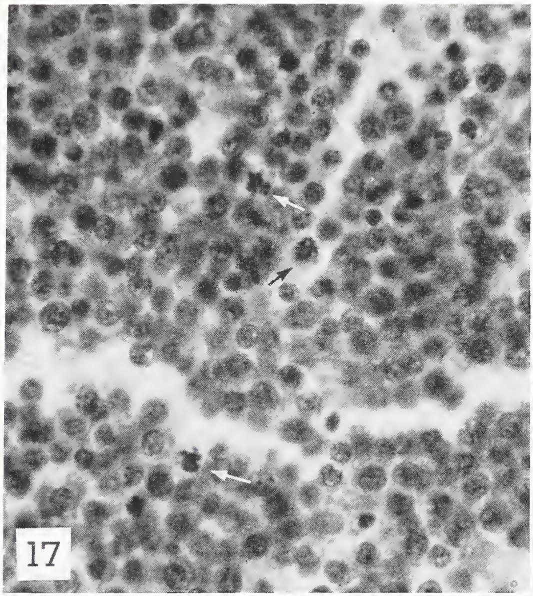
Feulgen-picro-methyl blue stain

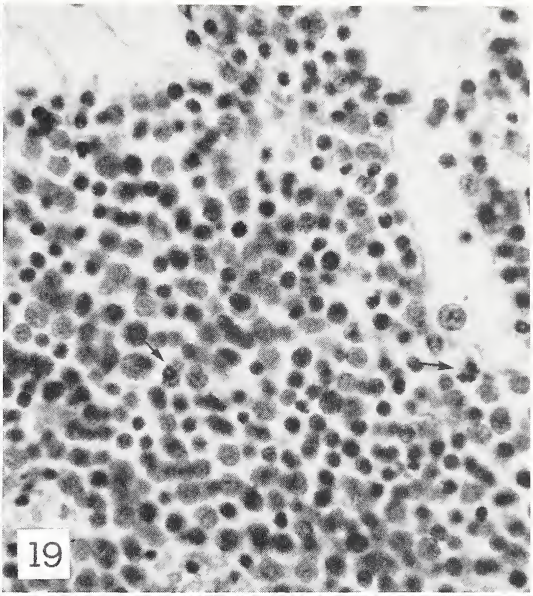
FIGURE 13.—Case 3: Mitosis in primordial epithelium of gonadal alveolus. × 900

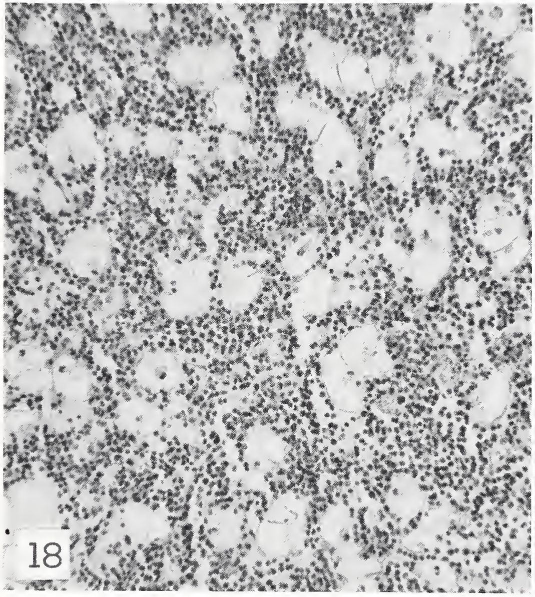
FIGURE 14.—Case 4: Neoplastic invasion of connective tissue. × 225

FIGURE 15.—Case 4: Invasive hyaline hemocytes. *Note* enlarged nuclei and pyknotic forms. *Arrows* indicate mitotic figures. × 900

FIGURE 16.—Case 5: Neoplastic invasion of connective tissue. × 225







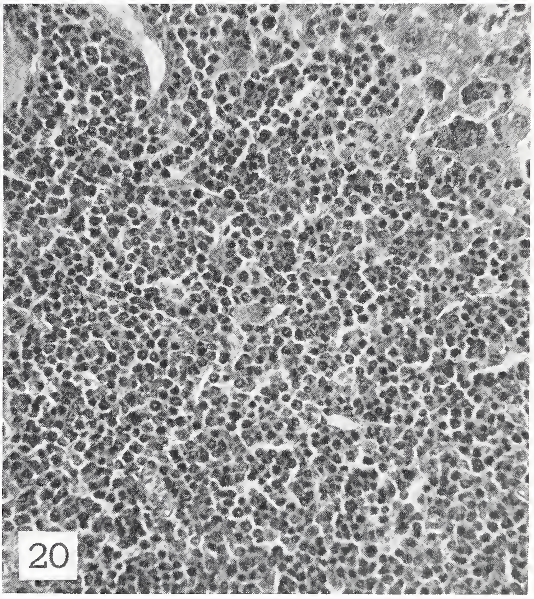


Figure 17.—Case 5: Neoplastic area in connective tissue. *Note* enlarged atypical nuclei. *Arrows* indicate mitotic figures × 900

Figure 18.—Case 6: Diffuse infiltration and neoplastic invasion of connective tissue. × 225

Figure 19.—Case 6: Neoplastic lesion showing mitotic activity (*arrows*) and pyknosis. × 900

Figure 20.—Section of hematopoietic neoplasm in liver of mouse. Harris' hematoxylin and eosin. × 225

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An Unusual Lesion in the Mantle of the American Oyster, *Crassostrea virginica*

J. A. COUCH,[1] *U.S. Bureau of Commercial Fisheries, Biological Laboratory, Oxford, Maryland 21654*

SUMMARY

A focal, tumor-like lesion in one of approximately 5,000 oysters examined from Harris Creek (a tributary of Chesapeake Bay, Md.) was examined histologically. The lesion replaced most normal connective tissue in the right mantle and consisted of relatively large basophilic blastoid cells with pleomorphic nuclei. The oyster cells in the lesion were atypical and many of the cells were in some stage of mitosis; up to seven mitotic figures were visible per oil immersion field. This high level of mitosis is atypical for normal oyster cells. It is not known whether these atypical cells are of hemic or of fixed mesenchymal cell origin. The oyster showed no evidence of infection by any recognizable parasite, and the condition was not comparable to known examples of non-neoplastic host response. Morphologically, the lesion strongly resembled some reticular neoplasms found in mammals, but because of the rarity of the condition and a lack of a complete natural history of the disease, only a descriptive comparison is made.—Nat Cancer Inst Monogr 31: 557–562, 1969.

HISTOLOGIC EXAMINATION of approximately 5,000 oysters from Harris Creek, a small tributary of Chesapeake Bay, Maryland, was made from 1964 through 1966, as part of a study of an epizootic associated with haplosporidan parasites. One of these oysters, a gravid female taken in June 1965, had a focal tumor-like lesion which occupied about two-thirds of the area in transverse sections of its right mantle. Unfortunately the oyster had been fixed, embedded, sectioned, and stained with Harris' hematoxylin and eosin before discovery of the lesion. Therefore the total extent of the lesion along the length of the mantle could not be determined, and the gross appearance of the affected tissue in the living oyster cannot be described. Histologic sections (7 μ) were taken from a larger, 1 cm block cut from the anterior portion of the oyster including the anterior gill region, stomach, and gonads.

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[1] I thank Dr. C. J. Dawe for his encouragement, pertinent suggestions, and review of this manuscript, and for sections of the mouse neoplasms in the illustrations.

HISTOLOGY AND CYTOLOGY OF NORMAL TISSUES

The surface epithelium of mantle and gills of the oyster was normal except for that small portion of the right mantle overlying the lesion. Connective tissue cells of the mantle and gills had a completely normal appearance (fig. 1). Gonads were fully developed and the follicles contained mature free and attached ova. The stomach epithelium was intact and unmodified. Normal amebocytes had infiltrated and accumulated in considerable numbers in the connective tissue next to the basement membrane of the stomach epithelium. A similar accumulation of normal amebocytes was around the intact intestinal epithelium. Concentrations of amebocytes in these epithelial areas did not appear to constitute a significant departure from the normal histology of amebocytes in other oysters of comparable size and development. The vascular sinuses of the body were normal, whereas those in the right mantle and one in the left mantle were quite abnormal, as described in the next section.

HISTOLOGY AND CYTOLOGY OF LESION

The right mantle was enlarged transversely because of the lesion (0.5–1 mm), but its vertical length (11 mm) was similar to that of the normal left mantle. In the lesion the tissue stained strongly basophilic as compared to the lighter staining adjacent normal connective tissue. The lesion's borders were poorly defined (fig. 2), and it extended from the proximal edge of the right mantle distally to involve about two-thirds the length of the mantle. The inner epithelium of the right mantle lobe was highly modified along the length of the lesion (fig. 5). The outer epithelium of this mantle lobe was unaffected. Stroma were absent around the lesion.

A single cell type was predominant in the lesion (figs. 3, 8, and 10). The diameter of the cells ranged from 4.9–12.6 μ, and their cytoplasm was relatively scant. Nuclei were large (diameters ranged from 3.5–10.5 μ), round, oval, or sometimes bilobed, and each had a conspicuous nucleolus (fig. 8). Rarely, cells of this size were binucleate (fig. 10), with moderately numerous and uniformly distributed division figures throughout the lesion [up to 7 mitoses per oil immersion field (fig. 9)]. The dividing cells were usually at metaphase or anaphase. For convenience, this cell type may be termed "blastoid" or undifferentiated. Vesicular cells of the connective tissue (*1*) present in the lesion were usually abnormally small and in many areas were replaced by the large blastoid cells. The lesion contained large (6.3 μ) and small (3.0 μ) pyknotic nuclei but necrotic areas were lacking.

Figure 4, from a blood sinus near the center of the lesion, is representative of the vascular sinuses in and near the lesion in the right mantle and is partially occluded by infiltration of the blastoid cells. Large blastoid cells were in a perivascular focus associated with one blood sinus of the opposite, otherwise normal, left mantle. This finding may represent a metastasis (if the term may be applied to molluscan pathology), or it could be interpreted as evidence of multiple foci of the disease. Normal oyster amebocytes apparently have the capacity to wander at random throughout the body of the oyster. Therefore, if the large blastoid cells were of hemic origin, they too may have been able to migrate through body vascular sinuses to other foci.

DISCUSSION

The origin of the blastoid cell type which makes up most of the lesion is inexplicable. If it is a blood cell, it has enlarged considerably and its nucleus is disproportionately large relative to the volume of its cytoplasm (fig. 10). Morphological resemblance is scant between this cell type and the normal or abnormal connective tissue (vesicular) cells of the mantle or body of the oyster. The relatively high number of dividing cells in the focal lesion is also unusual for postlarval oyster tissue. The division figures appear to be normal mitotic figures. No reliable chromosome counts or evaluation of chromosome structure could be made from these sections.

The blastoid cells in the oyster lesion morphologically resemble the cells of certain reticular sarcomas found in mice (*2*) and humans (*3*). This resemblance may be strictly morphological. Compare figures 5 and 8 of oyster with figures 6 and 7 of a "stem cell" type of reticular neoplasm from a DBA/2 mouse. The cells of the mouse sarcoma are considerably larger in nuclear diameter (16.0–24.0 μ) than the cells of the oyster lesion. Similarities are: 1) enlarged nuclei; 2) pleomorphic (lobed) nuclei; 3) occasional binucleate or multinucleate cells; 4) mitoses; and 5) focal rather than diffuse distribution.

The lesion cannot be precisely categorized because its origin is unknown and the course it would have taken in the living animal is unpredictable. Its morphological comparison with reticulum cell sarcoma in the mouse should not imply that it is necessarily similar in etiology or natural history to reticulum sarcomas in mammals. Characteristics of the lesion, such as enlargement of the oyster's mantle, presence of large blastoid cells undergoing mitoses, and replacement of normal connective tissue in the mantle, suggest that the lesion could reasonably be termed neoplastic.

At high magnifications (× 930), the lesion does not resemble host response (infiltration of amebocytes) to injury or parasites. No recognizable microparasites were found in the sections examined.

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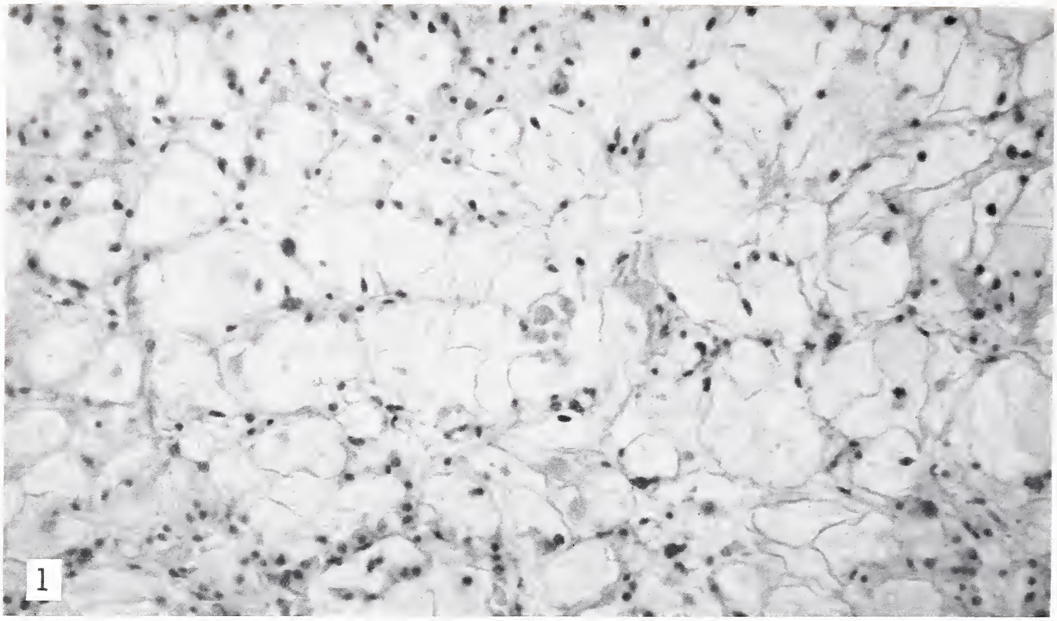
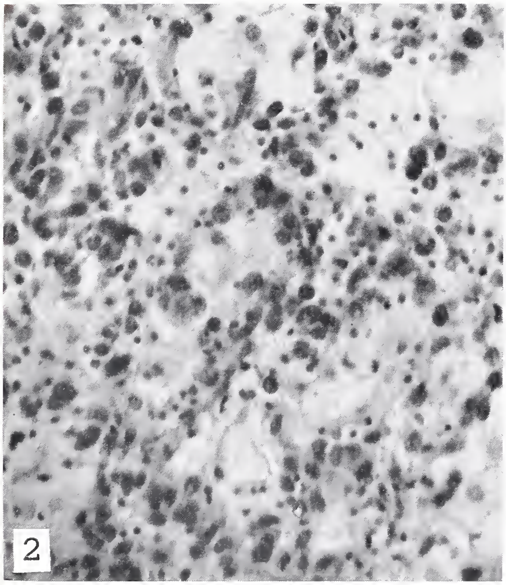




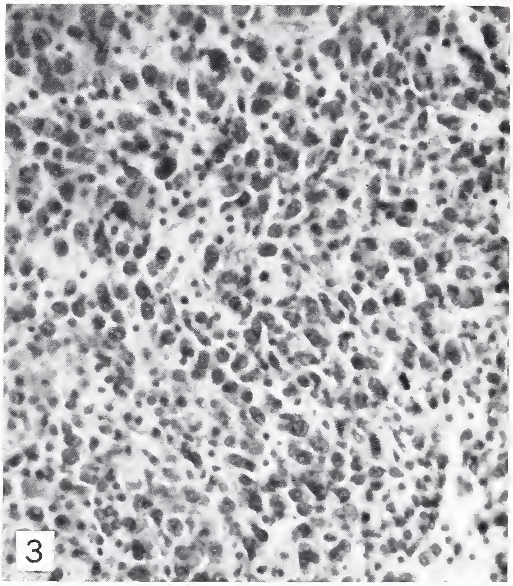
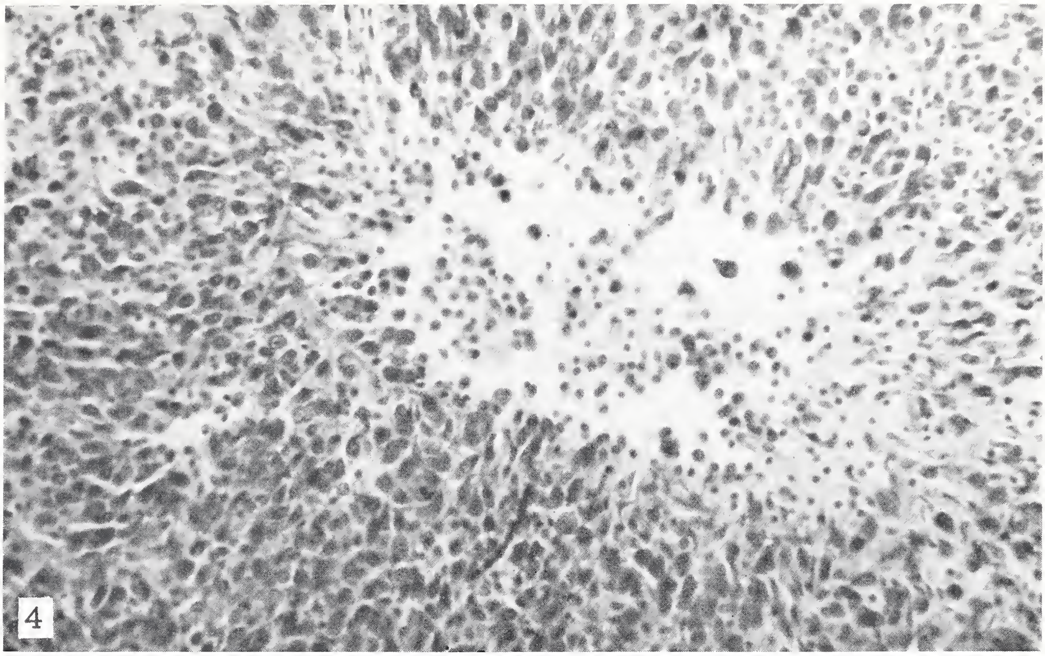
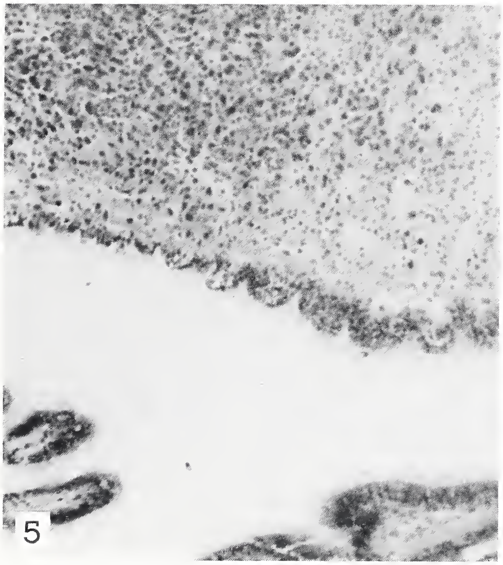


FIGURE 1.—Normal connective tissue of oyster. *Note* dark-staining nuclei of normal amebocytes in small blood sinuses between vesicular cells. × 430

FIGURE 2.—Connective tissue of proximal end of right mantle. *Note* large nuclei of blastoid cells on edge of lesion. Bulk of lesion is toward observer's *left* in figure. × 430

FIGURE 3.—Center of lesion in right mantle. Most cells are of the blastoid type. × 430





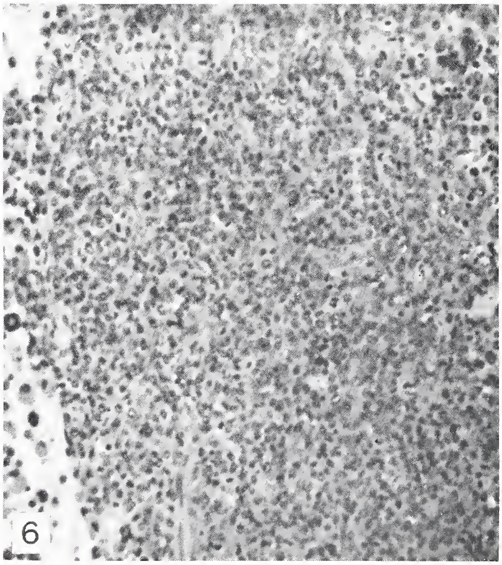


FIGURE 4.—Vascular sinus near center of lesion. Sinus is partially occluded. × 430

FIGURE 5.—Internal epithelium of right mantle. *Note* change in structure of epithelium from *right* to *left* along the length of mantle. Thickened portion of epithelium is normal (observer's *right*), whereas the portion of epithelium overlying lesion is abnormally thin and degenerate. × 100

FIGURE 6.—Portion of transplanted stem cell sarcoma from DBA/2 mouse. Compare with lesion of oyster in figures 3 (× 430) and 5 (× 100).

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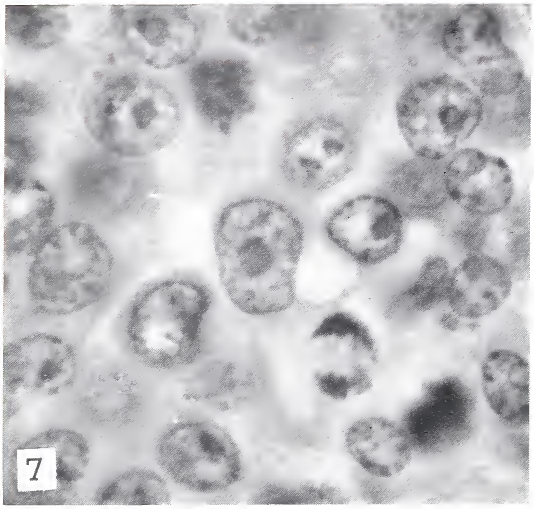

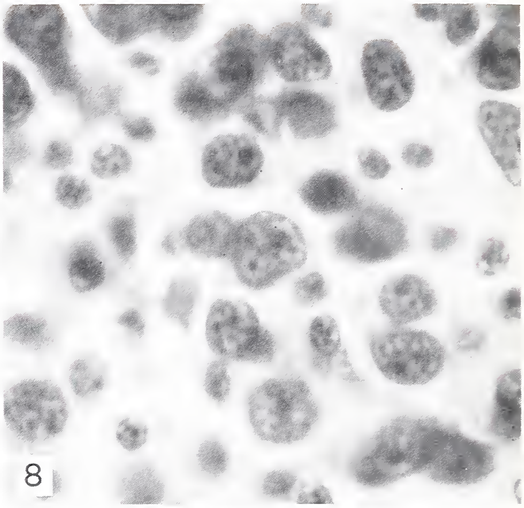

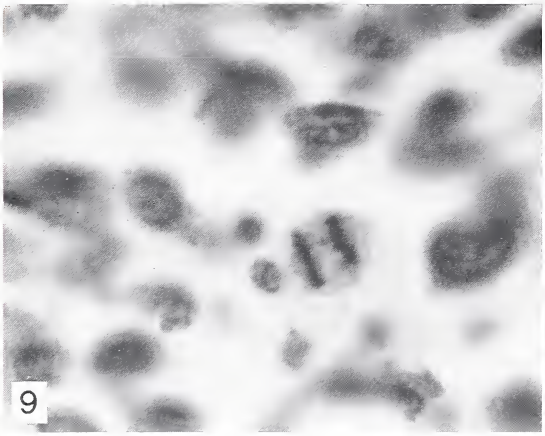

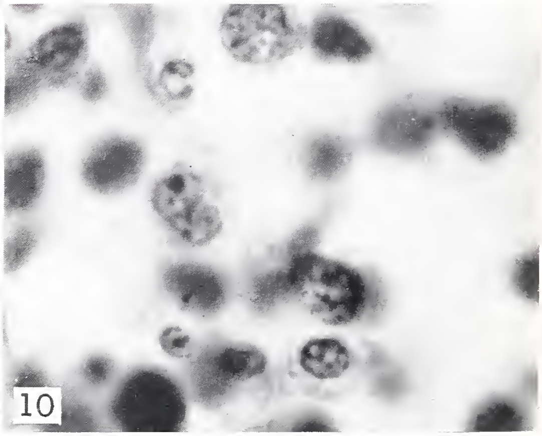

FIGURE 7.—Cells of stem cell sarcoma from mouse. *Note* pleomorphic nuclei and division figure (*center*). × 970

FIGURE 8.—Cells of lesion in oyster. Compare with figure 7. × 970

FIGURE 9.—Dividing cell in oyster lesion (anaphase). × 970

FIGURE 10.—Binucleate blastoid cell of oyster lesion (*center* of figure). × 970

Neoplastic Growth in Two Sydney Rock Oysters, *Crassostrea commercialis* (Iredale and Roughley)

PETER H. WOLF,[1] *Fisheries Branch, Chief Secretary's Department, Sydney, N.S.W., Australia*

SUMMARY

Neoplastic growths were found in the inner mantle of 2 specimens of the Sydney rock oyster, *Crassostrea commercialis* (Iredale and Roughley). One small tumor was spherical and had a slightly grooved surface 2 mm in diameter. Microscopic examination of the growth revealed columnar and cuboidal cells throughout; mucous cells and ciliated cells were confined to the outer region. The second tumor was oval, had deep ridges, giving it a "cauliflower-like" appearance, and was 5–7.5 mm in diameter. Microscopic examination showed ciliated columnar and cuboidal cells as well as mucous cells throughout the lesion. In both tumors, mitotic figures in anaphase, metaphase, and telophase were present. Both lesions showed intense inflammation, phagocytes were abundant in the cavities formed by the proliferating tissue, and there was necrotic tissue in the center of the lesions. The environment of the oysters collected and the materials and methods used are described.—Nat Cancer Inst Monogr 31: 563–573, 1969.

IN A REVIEW of the literature on tumors in invertebrates, Scharrer and Lochhead (*1*) noted the occurrence of tumors in mollusks. Williams (*2*) and Collinge (*3*) reported neoplasms in freshwater mussels (*Anodonta cygnea*) while more recently Pauley (*4*) described a tumorous growth on the foot of the freshwater mussel *Anodonta californiensis.*

In the American oyster, *Crassostrea virginica*, Ryder (*5*) and Smith (*6*) described benign, pedunculated, and nodular mesenchymal tumors, originating in the pericardium. Sparks *et al.* (*7*, *8*) described a similar mesenchymal tumor in the Pacific oyster, *Crassostrea gigas.*

During an inspection of an oyster shucking and packing plant on the Hawkesbury River, New South Wales, Australia, in September 1965, two

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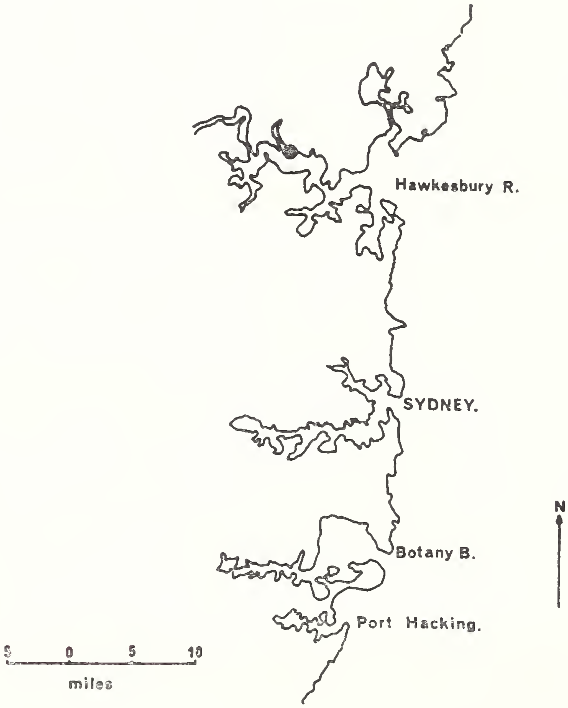
[1] I wish to express my gratitude and appreciation to Dr. Albert K. Sparks, University of Washington, and Dr. Margaret Lochhead, University of Vermont, for their advice; Mr. Gilbert Pauley, Battelle Memorial Institute, Dr. Clyde Dawe, National Cancer Institute, and Dr. John Harshbarger, Smithsonian Institution, for their examination of the slides and advice on the diagnosis of the tumors; Dr. G. E. Sullivan, Sydney University, for histological advice; Mr. Justice Myers and Mr. John Matthews, Sydney, for their photographic assistance; Miss Isobel Bennett, Dr. Ron Penny, and Dr. A. A. Racek, Sydney University, for reading the manuscript; Mrs. F. Hendy and Fisheries Inspector R. Cook for collection of the material.

Sydney rock oysters, *Crassostrea commercialis*, were found with tumor-like growths on the inner mantle adjacent to the inner lobe and anterior to the inhalant chamber. The packer reported that similar growths were noted from time to time, but not in great numbers.

AREA OF ORIGIN OF THE OYSTERS

The two affected oysters were from the Hawkesbury River system (text-fig. 1), one of the major oyster-growing areas in New South Wales. The system is primarily estuarine and oysters are cultivated in the intertidal zone on sticks, trays, and rocks. There are extensive mud flats and the system is normally subject to regular flooding. Between flood periods, the salinity in the main oyster-growing areas generally reaches 26 to 29 parts per thousand.



TEXT-FIGURE 1.—Map showing Hawkesbury River system.

Although the Hawkesbury River system is near Sydney, because much of the adjacent terrain is park land, the system has not been subjected to the normal consequences of urban development. Industrial and radioactive effluents are not discharged into the system and, as far as it is known, no neoplasm-inducing agents enter the river.

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MATERIALS AND METHODS

The two oysters were fixed alive in Davidson's fluid (95% ethanol, formalin, glycerine, glacial acetic acid, and seawater) and later photographed. The affected part of the mantle was then removed as far back as the gills, dehydrated, and embedded in paraffin. Serial sections, 7–8 μ thick, were cut and stained with Mayer's hematoxylin and eosin, Heidenhain azan (*9*) to show mucous cells (blue) and chromatin (red), Heidenhain hematoxylin, thiazin-picric acid (Heidenhain), and Fe-chloride-alizarine. Normal dehydration and mounting of coverslips with Canada balsam followed. Photomicrographs of appropriate structures were taken with normal light and contrast interference (Nomarski).

In late 1965 and early 1966, additional tumorous specimens were preserved by the Hawkesbury River packer but these had undergone postmortem decomposition and were not suitable for histological examination.

RESULTS

Although both specimens showed tumorous growths on the inner mantle adjacent to the inner lobe and anterior to the inhalant chamber, the growths were dissimilar in macroscopic appearance. In the first specimen, the tumor was small (approximately 2 mm in diameter), spherical, and had a slightly grooved surface (fig. 1). The tumor on the second specimen was considerably larger (5–7.5 mm in diameter) and oval with a deeply ridged surface (figs. 2 and 3).

The large tumor, shown in cross section in figure 4, appears to be epithelial in origin and has invaded the mantle by way of the middle lobe. The outer tissue comprises columnar and cuboidal cells interspersed with copious mucous cells (figs. 4 and 5) and appears similar to the tissue of the middle mantle fold. In the center of the tumor the number of mucous cells is greatly reduced and most of the remaining cells, which vary from tall columnar to cuboidal, are ciliated (fig. 9). Cavities, formed by ridges and folds of the proliferating lesion, are filled with numerous large phagocytes (fig. 6) indicating an intense inflammatory response by the organism. The tumor has an invasive appearance, replacing the Leydig's cells by infiltration rather than displacing and distorting them. Muscle tissue enclosed by necrotic tissue is shown in figure 7. Mitotic figures (fig. 8) in anaphase, metaphase, and telophase are taken to indicate the lesion was growing actively.

Photographs of sections of the small tumor are not presented, but examination of the sections has led to the conclusion that this lesion is merely a less advanced form of the large tumor described above. The point of origin is believed to be the epithelium of the mantle lobe and the microscopic structure of the lesion is essentially the same as that of the large tumor except that in the central portion the columnar and cuboidal cells lack cilia and mucous cells are absent. An intense inflammatory response is shown while numerous mitotic figures in anaphase, metaphase, and telophase are present.

The oysters were not examined for distant metastases.

DISCUSSION

The lesions described appear to be of epithelial origin. They are confluent with the epithelium and consist of ciliated columnar and cuboidal epithelial cells with mucous cells copiously interspersed. The central portions of the lesions appear highly compressed and highly cellular, with very densely staining nuclei. Invasion of the underlying connective tissue, engulfing of the pallial muscle bundles, disappearance of the blood sinuses, and mushrooming of growth, which rendered the 3 mantle lobes unrecognizable, indicate how deeply the lesions penetrated the available space. The "cauliflower-like" folding of the different cell layers obviously resulted from uncontrolled growth and subsequent lack of space. This is also confirmed by the complete deformation of the anatomy of the mantles, during which the conchiolin glandular tissues, blood sinuses, and Leydig's cells were pushed aside, to allow the lesions to expand. These tumors are unquestionably invasive.

Several tumors in marine and freshwater mollusks have been described in the past but, unlike the present ones, were mesodermal in origin and became externally visible only after internal pressure created pedunculated lesions. Their epithelial covering consisted of a single layer of relatively normal columnar cells [Harshbarger, personal communications (*10*, *13*)]. In the present cases, the growths were epithelial and invaded mesenchyme while simultaneously producing external outgrowths. Numerous mitotic figures in anaphase, metaphase, and telophase further confirm active cell proliferation. Mitotic figures are rare in adult mollusks and have been observed only by Butros (*11*). The work of Hillman (*12*) corroborates the paucity of mitotic figures in mollusks. Considering these facts, the present neoplasms seem to be indeed unique.

The main criteria for malignancy in mammalian tumors apparently are: cellular atypia, abundance of mitotic figures, invasion by proliferating cells, and distant metastases. On this basis, the oyster tumors could be classified as adenocarcinomas, but caution must be exercised since the definition of invertebrate neoplastic lesions is not precise and the nomenclature is still somewhat confused.

Dr. C. J. Dawe, National Cancer Institute, Bethesda, Maryland, Dr. R. Penny, Department of Medicine, Sydney University, and Mr. G. P. Pauley, Battelle Memorial Institute, Richland, Washington, examined several of the histologic sections and confirmed that the lesions are unquestionably neoplasms and appear to invade beyond the mantle muscle bundles (pallial muscle) into the Leydig's cells beneath. They concurred that atypia was evident in the center of the lesions and abundant mitotic

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figures were clearly present. The oysters were not examined for distant metastases. Because of lack of precedent, it is difficult to assign a standard diagnostic label to these lesions. Dawe (personal communication) suggests the term "papillary epitheliomas" as a sufficiently flexible tentative description, which would allow for later modification when the degree of invasiveness, lethality, and other biological characteristics, such as distant metastasis and transplantability to other oysters, have been studied.

The incidence of the neoplasms cannot be accurately assessed. The persons most likely to detect tumors are the professional packers, who open as many as 100 dozen oysters a day. Since the packer who provided the 2 specimens described detected only 9 more oysters similarly affected in 6 months, the incidence appears to be low.

After submission of this paper, another packer reported he had observed similar lesions in oysters from a particular shell bed on the Shoalhaven River (approximately 100 miles south of Sydney). He has since fixed in Bouin's fluid 12 oysters with one or more tumors, but the frequency of occurrence is not known because the number of opened oysters was not recorded. Three specimens were forwarded to the Registry of Tumors in Lower Animals, Smithsonian Institution, Washington, D.C., and the remainder will be investigated by the author.

The agent responsible for the tumors is not known. The fact that the 2 tumors examined and the 21 later detected by the packers were all in much the same position on the mantle suggests external injury was not the cause; it is unlikely that injuries would be restricted to one area. The inner shells adjacent to the lesions were normal and no irritative shell deformities were apparent. Pollution by chemical or radioactive carcinogens from industries can be ruled out, since both river systems do not take any industrial discharges. Further, the limitation of affected specimens to one particular area in the river system sufficiently proves this point.

Three more possibilities to be considered are: 1) Lack of oxygen: This is highly improbable, particularly in the Shoalhaven bed where tidal currents reach up to 2 knots velocity and oxygen must be abundant. 2) Virus infection: It also would be highly improbable that any infection would be confined to two very restricted areas instead of being spread over the major parts of the rivers. 3) Mutation: Perhaps the most reasonable explanation based on the little knowledge we now possess of invertebrate tumors is that normal invertebrate defense mechanisms, seemingly much more efficient in preventing tumors than defense mechanisms in vertebrate and mammalian organisms, were upset by genetic mutation, allowing tumors to appear in the genetically susceptible hosts.

However, before this explanation can be strongly supported, it will be necessary through future investigations to demonstrate whether superior defenses actually exist in mollusks and the importance of heredity versus environment in the maintenance of these defenses.

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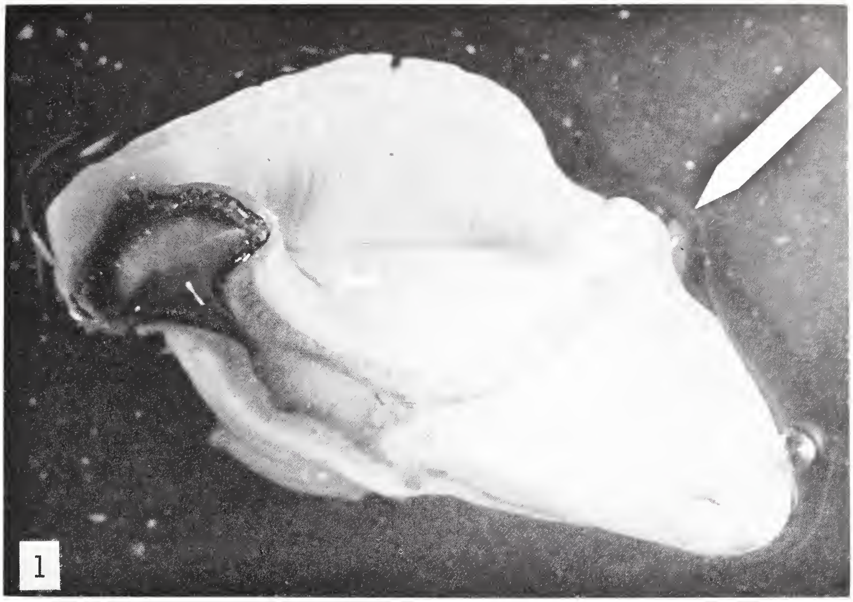

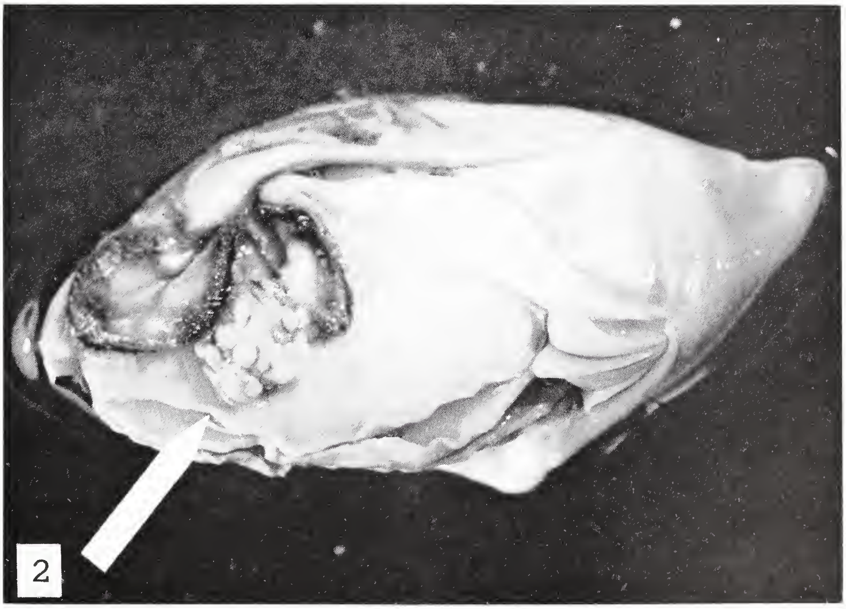

FIGURE 1.—Sydney rock oyster showing the *smaller* tumor on inner mantle lobes.
FIGURE 2.—Sydney rock oyster showing the *larger* tumor on inner mantle lobes.

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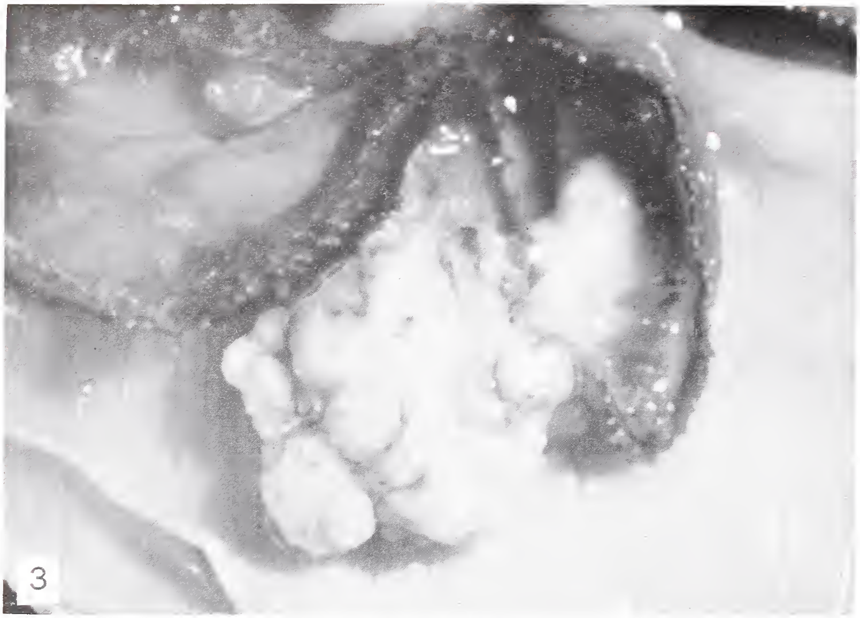


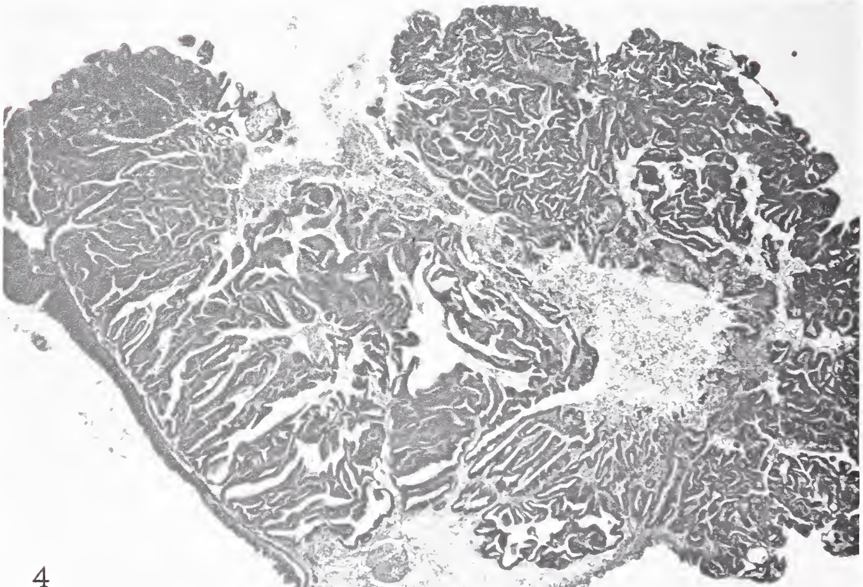
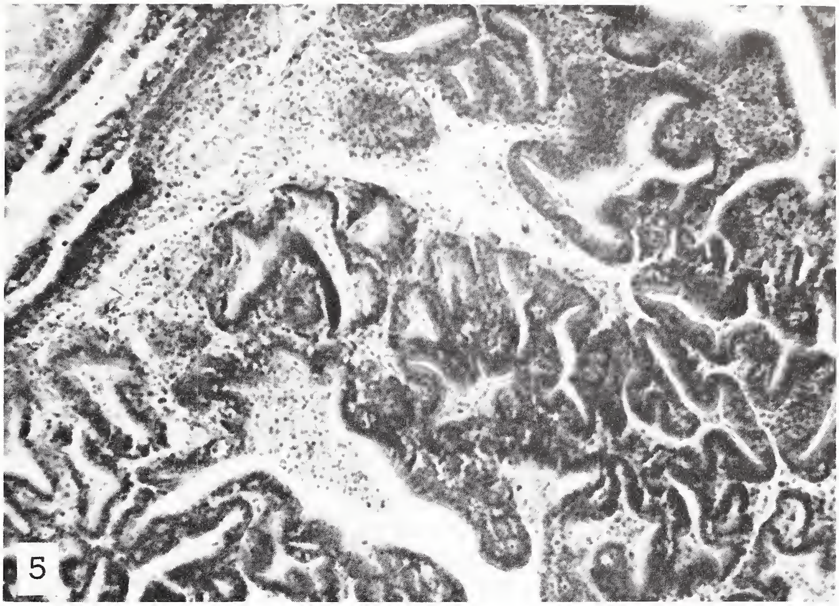


FIGURE 3.—Photographic enlargement showing external detail of the larger tumor.
FIGURE 4.—Section of the larger tumor showing general structure. Approximately × 15

Photograph by G. B. Pauley



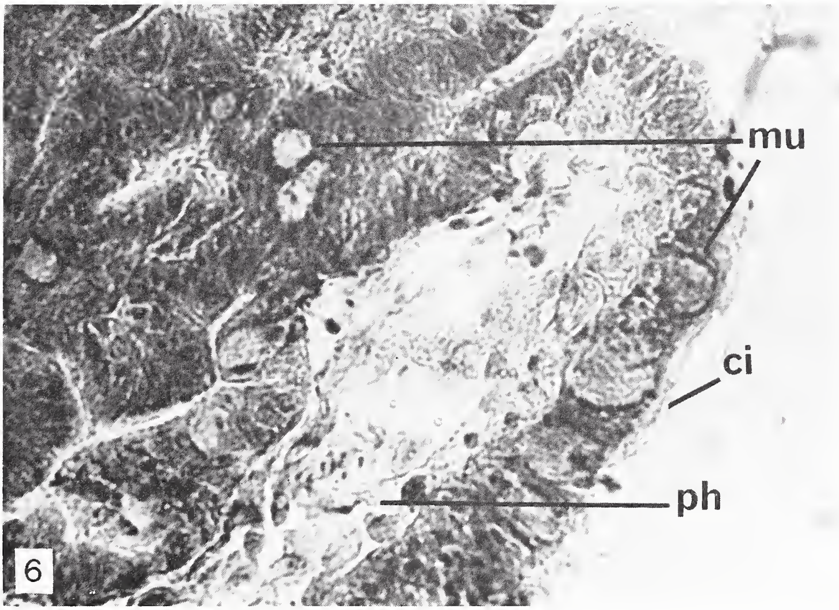


FIGURE 5.—Section of the larger tumor showing convoluted glandular type proliferation of epithelial tissue and phagocytes in cavity. × 320

FIGURE 6.—Section of the larger tumor showing mucous cells (*mu*), ciliated columnar cells (*ci*), and phagocytes (*ph*) in cavity. × 480

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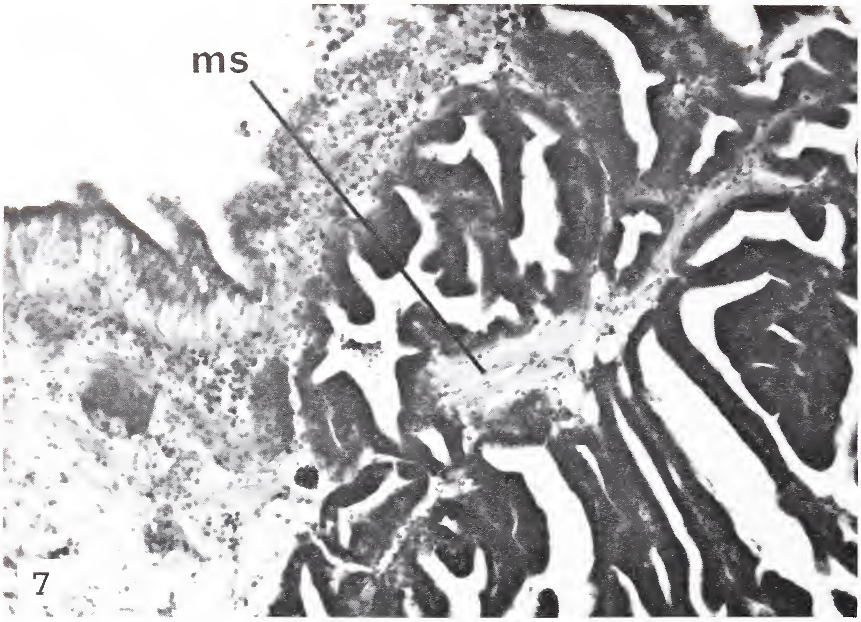

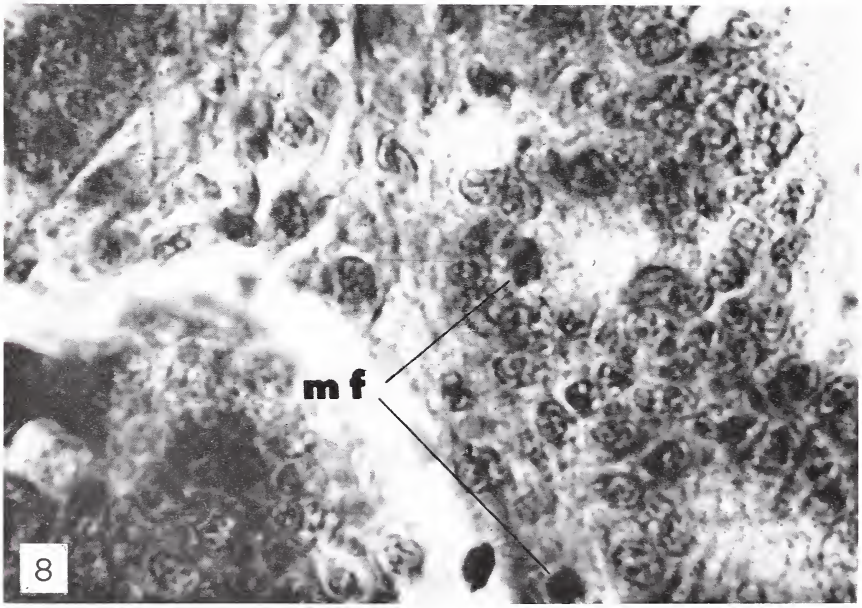

FIGURE 7.—Section of the larger tumor, showing pallial muscle (*ms*) surrounded by papillary neoplastic tissue. × 320

FIGURE 8.—Section of the larger tumor showing mitotic figures (*mf*) (oil immersion). × 840

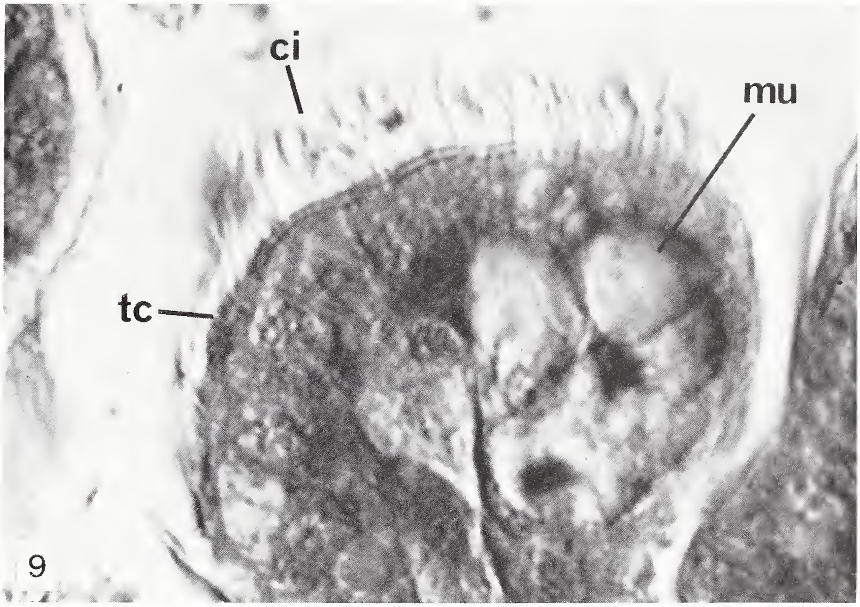

FIGURE 9.—Section of the larger tumor showing tall columnar (*tc*), cilia (*ci*), and mucous cells (*mu*) in *center* of neoplasmic lesion (oil immersion, Nomarski interference contrast). × 840

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Anomalies and Malformations in the Shells of *Crassostrea virginica*

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SUMMARY

The adaptability of *Crassostrea virginica* to different types of environment is associated with great variability in shell morphology. Anomalies are defined as deviations from common type in excess of normal variations. Malformations are harmful abnormalities that interfere with the functions of the organism. They were present in about 15% of shells collected along the east coast and Gulf of Mexico. Anomalies are not harmful and may be adaptive. They are noticeable as extreme elongations of oysters growing in mud, and are characterized by their reduplication of the shape of substratum by the attached valve, curved beaks, shell reversion, bifurcation of shell, regeneration, and sudden changes in the direction of growth. Malformations are not correlated to the environment. Some have characteristics of neoplasia. Others are reactions to infestations of boring organisms, parasites, and irritations caused by foreign bodies. Examples of malformations include massive globular pearls that occupy the greater part of shell space, pedunculated pearls, bridgelike structures consisting of a series of calcified globular units, calcified ridges over muscle scars, with tufts of periostracum on top, calcified cuplike cysts lined and covered with organic material, and recession of mantle resulting in excessive calcification of valves. The pearl-like malformations and cysts consist of irregularly arranged normal element of shell structure. They may be regarded as calcifying neoplasia. Unfortunately, soft tissues were not available for histological study. Nat Cancer Inst Monogr 31: 575–580, 1969.

FROM A MORPHOLOGICAL point of view the shells of *Crassostrea virginica* are highly plastic, varying in configuration, size, thickness, sculpture, and pigmentation. The species easily adapts to various environmental conditions, often reflecting the configurations of hard surfaces to which they happen to attach, or the character of bottom sediment on which they grow unattached. When they develop on soft mud or crowded clusters, their shells are usually long and slender, with sharply curved beaks for better anchoring. Those attached to round rocks or irregularly shaped solid objects faithfully reproduce the configuration of the substratum.

During my investigations in the coastal waters of the United States from 1925–1958, I examined 8,000 oyster shells, of which nearly 250 were abnormal. These abnormalities may be grouped into two categories: 1) anomalies, which do not interfere with the functions of the organism and apparently are harmless, and 2) malformations, in which normal activities are presumably hindered by the pathological shape or structure of the valves. Allowing for the great variability in shell morphology, only those specimens are considered anomalous or malformed in which the morphology of the shell exceeds the range of normal variability.[1]

Anomalies.—The following anomalies are described in the order of frequency of their occurrence.

1) Changes in direction of the principal axis of growth. Normally the shells of very young oysters (spat), shortly after their attachment, are circular. Soon, however, the valves curve slightly to the left (viewed from the right side) and become asymmetrical. In some shells the established direction of the principal axis of growth does not remain constant. A shift of a 90° angle was found in 2- or 3-year-old oysters; a number of older mollusks were found in which a less pronounced shift in axis suddenly occurred and could not be associated with any visible obstruction.

2) Bifurcation of shell. This anomaly results from sudden appearance of two principal axes of growth separated by an angle of about 60° (text-fig. 1). Bifurcation of shell is accompanied by the elongation of the muscle scar in the anterio-posterior direction. Gunter describes such a unique case of the bifurcation of the adductor muscle found in a Davis Bay, Mississippi, oyster (*1*). Bifurcation of shell was found in the oysters from different areas and was not associated with geographical distribution or with obstruction to their growth.

3) Stairway-shaped valve. As a result of periodic interruptions in the rate of growth of shell along its periphery and continuous calcification of older parts, the valve resembles a series of steps ascending from the ventral edge toward the hinge. Conditions responsible for this abnormality are not known.

4) Overcalcification of the right valve. Normally, the rate of calcification is about 2–6 times greater on the left (lower) valve than on the right (*2*). Consequently, the right (upper) valve is usually thinner and lighter than its counterpart. In several very old oysters from Maine, New Hampshire, and Connecticut, the right valves were considerably thicker than the left (text-fig. 2).

5) Inversion. Complete inversion of hinge structure and its interlocking teeth was described for several freshwater and marine bivalves. This phenomenon, resembling "*situs inversus*" of higher animals, was named heterotaxy by its discoverer, Moquin-Tandon (1855). Haas (*3*) has adequately reviewed his work and many other cases of heterotaxy in bivalve species, including the tertiary genus *Goodalia* mentioned by Dall (*4*) and the so-called "sinister" shells described by Lamy (*5*). In all these

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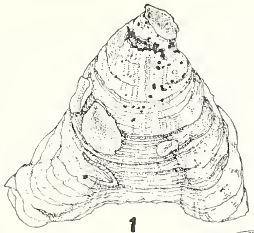
[1] Representative specimens are deposited with the Registry of Tumors in Lower Animals, Museum of Natural History, Smithsonian Institution, Washington, D.C.

Drawings reproduced in text-figures 1 through 8 were made from nature by Mrs. Ruth Von Arx, Woods Hole, Massachusetts.



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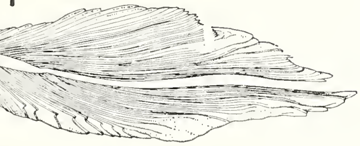

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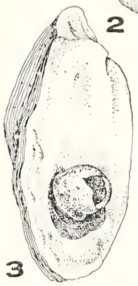

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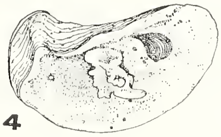

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Text-figure 1.—Bifurcation of shell in an oyster from Matagorda Bay, Texas (5.7 × 7 cm).

Text-figure 2.—Abnormal thickness of the right valve of an old Maine oyster (22 × 4.5 cm).

Text-figure 3.—Large cyst partially covered on top with calcified material. Maryland, 1944 (10 × 4 cm).

Text-figure 4.—Massive deposit of calcite material of irregular shape, Five Fathom Creek, South Carolina (5.7 × 3.2 cm).

instances, the structure of a valve was a mirror-like replica of its opposite member. No complete inversion was found in the shells of *C. virginica*, but in a number of specimens, the valves, viewed from the right side, curved to the right instead of to the left (*6*). This change in the direction of curvature did not affect the hinge part, which retained its normal left or right structure.

Malformations.—According to the definition given above, these abnormalties are pathological. They are associated with the disturbance of calcium metabolism which manifests itself in an overcalcification of selected parts of the organism. It is a well-established fact that the formation of shell, including its sculpture and pigmentation, is the function of the mantle. Under the impulses originated from the circumpallial nerve and conveyed through the radial nerves and their ramifications, the mantle periodically extends forward and then withdraws inside the shell cavity. By contracting some of the radial muscles, the mantle folds in temporary ridges, while the epithelial cells of the outer lobe secret conchiolin and deposit calcium salts over the newly laid organic matrix. The position of the mantle is governed by muscular contraction, but the secretion of shell materials depends on the concentration and activity of various enzymes. It is known from the work of Wilbur (*7*) and Glimcher (*8*) that several enzyme systems are present in the calcifying tissues of bivalves. They can be demonstrated by proper histochemical methods (*9*). Saleuddin (*10*) found that in the freshwater mussels, *Anodonta grandis* and *Lampsillis luteola*, the epithelial cells of the outer fold contain appreciable amounts of RNA which, after injury, at first decrease markedly and then show a significant increase. The amounts of alkaline phosphatase also increase after shell injury.

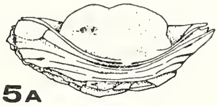
Physical injuries to shell and the underlying mantle of *C. virginica* are rapidly repaired by the deposition of layers of calcified material covering the injured area, sealing the holes bored by various animals and plants, and forming protective partitions or vertical walls that fill up the gap in the valves. Oysters severely damaged by dredging or tonging are often found on the commercially exploited oyster beds. The nature of their injuries is easy to recognize.

Malformations associated with conditions not related to the outside environment are of special interest to the student of oyster pathology. They fall into two classes: 1) cysts and calcified cups; and 2) calcified tumors and pearl-like structures attached to the valve or formed inside the soft tissues. Unfortunately there was no opportunity to study the histopathology of these formations. Occasionally they were located near the holes made by boring organisms, thus suggesting that irritation coming from the proximity of an invader was responsible. In other similar cases the effects of boring organisms were not apparent.

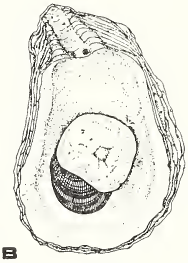
1) Cysts and calcified cups. Progressive phases of cyst or cup formation can be seen in a series of shells in the collection. The beginning is usually marked by semitransparent patches of newly deposited shell material which form a blister over an area damaged by boring. Black mud often accumulates under the blister. As the malformation grows in height and width, it covers larger portions of the inner surface of the valve and displaces the tissues and organs above it. Eventually a well-calcified and oval-shaped capsule or cup is formed. The most favored location is on the muscle scar or in the areas of the valve near it. Text-figure 3 shows a large calcified cyst about 1.5 cm high and 2.4 cm wide at the base. The top is covered by black material which also lines the walls of the cyst. The lining is made of calcified organic matrix, in places resembling the structure of the prismatic layer of shell. The inside of the cyst is filled with a debris of tissues, blood cells, numerous bacteria, and other microorganisms, probably representing secondary infestation. Cysts or capsules of the described type are common in the adult oysters of the Chesapeake Bay and estuaries of the Southern and Gulf States. Because of their size and position, it is apparent that they are of pathological origin, probably identical with the malformations associated with the so-called shell disease of *Ostrea edulis* in Holland. Dutch oyster growers attribute catastrophic mortalities of their oysters to an unidentified fungus in the cysts. There is no evidence that shell disease constitutes a serious problem in the populations of *C. virginica.*

2) Calcified tumors and pearl-like structures. Massive calcification of selected areas of shell and the formation of large pearl-like structures rarely occur in *C. virginica.* Only eight specimens with these malformations were found in the collection. In five of them the irregular deposits, laid over the inner surface of the valve, extended vertically as peaks and valleys (text-fig. 4). In the three others, smooth and rounded deposits

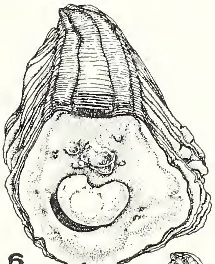
reached huge dimensions in relation to the size of the oyster. The largest measured 2.5 × 3.0 × 1.8 cm and occupied an extensive area of the valve near the muscle scar (text-fig. 5a, s). The oyster was only 8 cm long, and its age could not be determined by the number of rings on the shell, which was heavily infested by boring clams. On the cross section, the entire structure appeared to be made of a solid mass of calcite lamellae laid between the conchiolin sheets. The posterior and anterior edges of the valves were greatly thickened by the deposition of calcite-ostracum. The proximity of the boring clams to the inner surface of valve suggests that excessive calcification may have been induced by the boring organism.


5A

Text-figure 5a.—Single large, round deposit on a shell of Matagorda Bay, Texas, oyster. Pronounced thickening of the edges of the valve infested with boring clam. Valve cut transversally. Dorsal half viewed from the right side (4 × 3.5 cm).


5B

Text-figure 5b.—Same oyster, ventral half, viewed on cross section. *Note* thickening of the valves' edges.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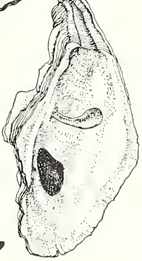
Text-figure 6.—Old oyster with abnormally long beak and deeply cupped valves; large deposit over the muscle area and numerous smaller deposits on the dorsal part of the valve. Chincoteague Island, Virginia. Winter 1938 (10.5 × 5.2 cm).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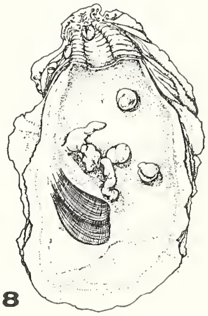
Text-figure 7.—Pedunculated pearl in the shell of an oyster from Texas waters, courtesy of Dr. G. Gunter (8 × 4 cm).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Text-figure 8.—Multiple pearls forming a bridgelike structure inside an oyster from Chincoteague Island, Virginia. Winter 1938 (12.3 × 5 cm).

One monstrous oyster contained one large deposit and several small ones on each valve (text-fig. 6). The beaks were disproportionately long, measuring 4 cm, while the overall length of the shell was only 10.5 cm. The oyster was deeply cupped. Thick and heavily infested valves were laid in a series of rings which indicated an age of 20 years or more. Approximately the same number of growth lines could be counted on the inner surface of the hinge. Apparently the monstrosity originated from the failure of the mantle to extend outward during the growth periods while the secretion of shell material and its mineralization proceeded at the normal rate.

It is a well-established fact that true pearls are formed in the tissues of pearl oysters as a result of local irritations caused by grains of sand, pieces of polished shells artificially introduced in shell-secreting tissues, and infestation by parasitic worms. Edible oysters produce no true pearls but occasionally contain globular nodules or concretions of calcite lacking luster. An interesting case of a "pedunculated pearl" was found in a Texas oyster which an oysterman gave to Gunter. As can be seen in text-figure 7, a fairly large pear-shaped nodule is supported by a long, curved stem. Judging by the position and the length of the peduncle, the "pearl" was formed in or near the visceral mass.

In another rare specimen (text-fig. 8), several globular or irregular "pearls" were found cemented together in a grotesque bridgelike structure extending across the body of the oyster. A large blister was near the area of the origin of this structure. The right valve of the shell was missing. It is impossible to determine what specific conditions were responsible for the formation of this unique structure.

Apparently large calcified masses in the body of an oyster interfere with its normal functions. Malformations of this type resemble calcified neoplasms found in other animals; in some cases they probably developed as a response to irritations caused by the proximity of boring and shell-invading organisms. It is reasonable to assume that the described malformations represent a phenomenon analogous to the over-calcifications of various selected part of mammals described by Selye (*11*) under the general term calciphylaxis.

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Mercenene: An Antineoplastic Agent Extracted From the Marine Clam, *Mercenaria mercenaria* [1]

SISTER ARLINE C. SCHMEER, *O.P.*,[2] *Institutum Divi Thomae Research Laboratory, Department of Biology, Ohio Dominican College, Columbus, Ohio 43219; and the Marine Biological Laboratory, Woods Hole, Massachusetts 02543*

SUMMARY

Antineoplastic activity of an agent (mercenene) extracted from marine clams (cherrystone, quahog), *Mercenaria mercenaria,* has been assayed *in vivo* against several well-established mouse tumors, and *in vitro* on the HeLa (Atl) and normal human amnion (FL) cell lines. Anticancer effects on transplanted subcutaneous tumors were most significant in fractionated samples purified on gel filtration columns. In both the Sarcoma 180 and Krebs-2 carcinoma, tumor regressed completely in at least 70% of the treated animals. The dosage schedule consisted of reconstituted lyophilized samples of mercenene each day for 7 days. No toxicity was observed in the dose and concentration administered to achieve tumor regression or inhibition in CF_1 female, 4- to 5-week-old mice. Control animals died in 21–28 days, whereas treated animals survived until killed 6 months after initial treatment. All cells died in cultures of HeLa and KB lines within 48–72 hours after treatment. No cytotoxicity was observed on the amnion line. Chemical characterization of mercenene confirms that the active principle is a species of low molecular weight, bound rather loosely to a macromolecule which is a protein or glycoprotein. Studies have been completed to determine the kinetics of the cellular proliferation of the Krebs-2 carcinoma. Data from these investigations will determine the basic parameters for investigating those changes induced in the cells of this tumor by any chemotherapeutic agent. This should then elucidate the regulatory mechanism involved in mercenene's antineoplastic effectiveness at the cellular level.—Nat Cancer Inst Monogr 31: 581–591, 1969.

THE FACT that cellular extracts of many origins have both a stimulating and inhibiting effect on cancer cells has been known for a number of years in many laboratories. Kelly and McDowell (*1*) have reported such data.

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[1] This study has been partially supported by grant T–361 from the American Cancer Society, Inc.; Public Health Service Fellowship GM–34,903 from the National Institute of General Medical Sciences; The Ashland County Cancer Society and the Ashland County Health Foundation, Ashland, Ohio, and the Tad Beck Fund, Lincoln, Neb.

[2] I wish to express my sincere appreciation to Mrs. Grace Beery, research assistant, Department of Biology, Ohio Dominican College (formerly the College of St. Mary of the Springs), Columbus, Ohio, for her assistance in the cytological studies of this investigation.

The anticancer activity of normal tissue extracts from spleen, brain, and liver was reported by Schroeder and Cook as early as 1939 (*2*). Heilbrunn *et al.* (*3*) suggested that tissue extracts of mollusk ovaries had an antimitotic influence on developing marine eggs. Nigrelli (*4*), Li (*5*), and Gerber *et al.* (*6*) presented evidence that the ocean and its fauna and flora are sources of antimicrobial agents. Sullivan and Nigrelli (*7*) attributed antitumor activity to a species of sea cucumber.

In 1962, while searching the literature for mention of a marine invertebrate species to be used as a tumor donor, the author could not find a verified cytological reference that malignancies occurred in these animals. The question was then asked if this was due to lack of data or possibly to some antineoplastic principle shared by these animals. The second hypothesis was tested experimentally when preliminary studies were made with crude aqueous tissue extracts of several marine mollusks (*8*, *9*). This group was chosen because it is a major source of food, is available in large numbers, and can be handled with comparative ease.

This paper reports the subsequently found presence of an anticancer agent (mercenene) found in mollusk tissues and in particular in the common marine clam (cherrystone or quahog), *Mercenaria mercenaria.*

METHODS AND MATERIALS

In vivo assay for antineoplastic activity.—CF_1 female mice, 4–5 weeks old, with a mean weight of 18 g received subcutaneously in the right axillary region approximately 3×10^7 Krebs-2 (K-2) ascites cells. These cells were from 8- to 12-week-old CF_1 females who had received 3×10^7 ascites cells intraperitoneally a week before harvesting. Four days after subcutaneous implantation, a palpable solid tumor with a diameter of 5–8 mm was seen in 100% of these animals. The mice, chosen randomly, were divided into experimental and control groups of 10–15. For all results reported with use of the partially purified extracts, each experimental mouse received subcutaneously in the left axillary region 0.25 ml mercenene, each day for 7 days. Control animals received 0.25 ml placebo of sterile physiological saline. On day 8, the tumors were excised from all groups and weighed. The ratio of combined tumor weights for the two groups (T/C) was taken as the index of extract activity.

In vitro assay for antineoplastic activity.—This assay utilized the HeLa (Atl) and human amnion (FL) cell lines. The cells were grown in monolayer on sterile glass in milk dilution bottles with Eagle's basal medium, 10% calf serum, and Hanks' balanced salt solution (BSS). After 7 days, they were trypsinized and centrifuged at low speed for 7 minutes by standard sterile techniques. The resultant pellet was resuspended in Hanks' BSS at a 1:200 dilution. Fractions of 1 ml, containing approximately 10% calf serum, and Hanks' balanced salt solution (BSS). After 7 days, 24 hours at 37°C. After 24 hours, 0.10 ml, approximately 2–3 mg dry weight of extract purified on Sephadex gel columns, was added to each Leighton tube. These tubes were then reincubated at 37°C, and readings taken at 24, 48, and 72 hours by the method of Syverton *et al.* (*10*). The monolayer was stained with May-Grünwald-Giemsa.

Preparation of crude aqueous extracts of mercenene for several mollusk species.—Tissue extracts of fresh, live specimens of *Mercenaria* (clam), *Ostrea v.* (oyster), *Busycon c.* (channel whelk), *Helix* sp. (snail), and *Loligo* sp. (squid) were prepared by removing the animals from their valves. Each species was homogenized separately. One gram of this mixture was added to 100 ml sterile water and allowed to stand in a separatory funnel; the remaining sediment was removed by low-speed centrifugation. The supernatant was stored for test purposes. Toxicity tests were set up as follows: 100% supernatant and dilutions of 1/10, 1/20, 1/40, and 1/80 supernatant to sterile water. One ml samples of these dilutions were given intraperitoneally each day for 7 days. During this treatment, a toxic effect was never noted. In a succeeding experiment, a selected dilution for each extract was then administered using the dosage schedule described earlier.

Preparation of purified samples utilizing Sephadex gel filtration columns.—Initial studies on the crude aqueous extracts led to precipitation with ammonium sulfate and purification on Sephadex (*11*). Utilizing the molecular sieve technique, it was determined that 60–70% of the active agent was recovered with the G-25 and G-10 gels. Both whole-body and individual tissue extracts were made of the clam and treated as previously described (*8*). The supernatant was applied to the various gels, G-100, G-75, G-50, G-25, and G-10. A dosage schedule, identical to that used with the crude extracts of *M. campechiensis* and described under "Methods and Materials" ("*In vivo* assay . . ."), was followed in this series of studies.

Longevity studies.—A series of animals was treated with mercenene for 7 days, but the mice were not killed on the 8th day. Six months later mice from this study were autopsied and examined for the presence of tumor and tumor cells.

RESULTS

Crude, Aqueous Extracts

Squid, snails, and oyster gave approximately the same results. Whelk had the least amount of antitumor activity. The clam showed the greatest amount of antineoplastic activity (*9*).

Purification on Sephadex (Table 1)

In the gels (G-25 and G-10) of greatest recovery, the active agent was present in the first 100–400 ml from the columns (*8*, *11*, *12*) (text-figs. 1 and 2; fig. 1).

Table 1.—Effectiveness of mercenene against the Krebs-2 carcinoma in mice*

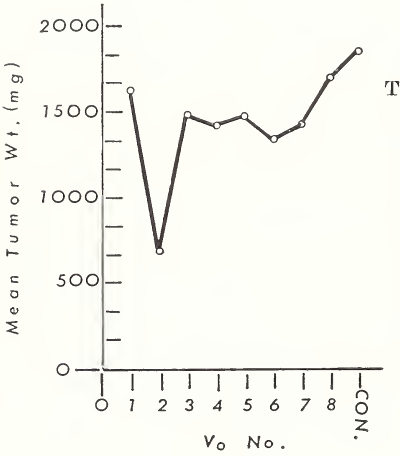
MTW ± SE† (mg)	MTW ± SE‡ (mg)		Mean survival§		
	Untreated	Treated	Days	Untreated	Treated
450 ± 38	2450 ± 125	620 ± 49	8	45	45
			28	0	42
			180	0	41

*The values are averages of 3 experiments, 1-3: A total of 180 CF_1 female mice (15-18 g) was used; subcutaneous treatment with mercenene was begun 4 days after tumors were implanted in 75 animals taken at random; 75 mice served as controls; 8 mg of mercenene was given each day for 7 consecutive days.

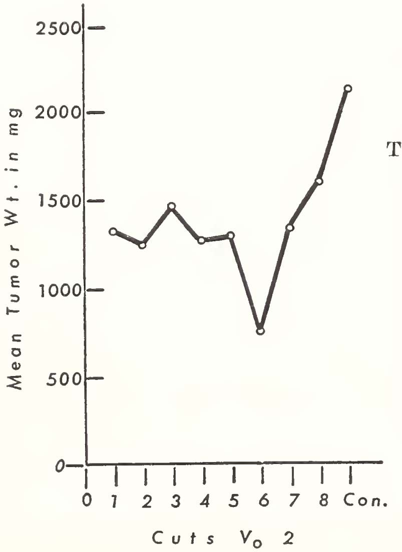
†MTW = mean tumor weight; SE = standard error. Four days after implant of tumor and before treatment was begun, 30 mice were killed, and tumors excised, weighed, and studied histologically.

‡After a 7-day dosage schedule, 30 mice from the 75 in the treated group and 30 mice from the control group of 75 were killed, and tumors were excised, weighed, and studied histologically.

§After 180 days, the surviving mice were killed.



Text-figure 1.—Gel filtration of mercenene (Sephadex G-25): The regression and inhibition of K-2 tumor growth following the dosage schedule described. V_o = a void volume of pooled fractions of 10 ml/fraction. One V_o = 80 ml for the column used. V_o No. 2 contained the greatest amount of antitumor activity (mean tumor weight for this experiment was 690 mg compared to control tumor weight of 1863 mg). Figure 1, p. 589, shows excised tumors.



Text-figure 2.—Fraction or cut No. 6 in void volume No. 2 (V_o 2) contained the most effective concentration of antitumor activity after filtration on G-25 Sephadex. Individual fractions were given to the experimental mice instead of pooling the samples as seen in text-figure 1. Each *point* represents the mean weight of tumor for 15 animals. Experimental mice had a mean tumor weight of 750 mg. Control tumor weight was 2250 mg.

A typical experimental result gave a mean control tumor weight of approximately 2250 mg and an experimental mean weight of 750 mg (text-fig. 2). The light level study of tissues prepared from tumor samples confirmed these results (*13*).

In histological study of control groups receiving saline, sections of the untreated tumors *in vivo* showed large areas of undifferentiated neoplastic cells. The sections exhibited polychromatophilia of tumor cells and evidence of cytoplasmic degeneration, including vacuolization, loss of nuclear and cytoplasmic staining properties, and accumulation of lipid material. A high nuclear-cytoplasmic ratio was evident; both pleomorphic nuclei and nucleoli were present. Giant cells and many mitotic figures were evident at appropriate magnifications in many fields. Areas of extensive cellular degeneration showed a breakdown of cell structures, including loss of discrete nuclear staining characteristics. There was evidence of loss of cellular membranes in areas composed of necrotic tissue, cellular debris, and granules. The picture was that of excellent oncolysis.

By contrast, the microscopic appearance of the tumor sections from mice treated for 7 days was one of extensive oncolysis. The tumor tissue was infiltrated by collagenous fibers which were accompanied by invading fibroblasts. In some areas of fibroblasts there were mononucleated cell types, such as lymphoid cells, histiocytes, and plasma cells. Small areas of shrunken muscle nuclei and fiber cells were also evident after 7 days of treatment. Vascularization was good, and fewer irregular nests of malignant cells were evident when compared to the controls (*8*, *9*).

At autopsy of all 41 surviving animals in the longevity study, 6 months after tumor implantation and 7 days of treatment with more purified extract, young scar tissue (dense fibers) was seen, but no tumor cells persisted. Very few shrunken pyknotic muscle cells were evident.

Syverton's scale of cell degeneration was used in determining *in vitro* effects of the clam extract on HeLa (Atl) and human amnion (FL) cell lines (figs. 2 and 3). Repetition of this experiment 8 times gave approximately the same cytotoxicity. Little to no toxicity was observed in the treated amnion cultures, while in the treated HeLa tubes the cells were completely degenerated within 48 hours. All HeLa cells seemed equally susceptible to the extract. Control cultures of HeLa (placebo of physiological saline) were normal.

The clam tissues with the most promising tumor-inhibiting activity were the crystal body and the liver (*14*). The results of studies involving these tissues are known to at least equal the regression and inhibition noted in animals treated with the whole clam body when an identical dosage schedule was used. Studies are presently under way to characterize chemically the antitumor agent both in liver and in the crystal body.

Chemical characterization of mercenene, utilizing the samples from gel filtration, suggests the agent may be either 1) a mixture of two or more substances, one readily dialyzable and the other(s) not so, or 2) a homogeneous species of such molecular weight that it passes slowly through cellophane membrane. Still a third possibility is that the active principle is a species of low molecular weight, bound rather loosely to a macromolecule which is a protein or a glycoprotein (*12*).

Analysis of desalted samples of mercenene was followed by assay in cell culture with the HeLa line (table 2). The net loss of biological activity on desalting was negligible, while this procedure provided approximately a tenfold concentration of activity (*12*).

TABLE 2.—*In vitro* biological activities for a typical mercenene fractionation

Sample*	Weight (mg)	Activity†
Dried samples from crude tissue homogenate‡	2200	§
Mercenene, purified by Sephadex fractionation§	488	§
Desalted mercenene	66	0. 005
Dialyzate from desalted mercenene	53	0. 004
Desalted, exhaustively dialyzed mercenene	4	0. 002

**Mercenaria mercenaria*, collected at Woods Hole, Mass., water temperature 18–22°C.

†Maximum dilution, in mg/0.25 ml, at which human HeLa cells display 4+ degeneration.

‡Extract from approximately 1 clam, which had been processed [*see* (*2*)] but not fractionated on Sephadex.

§The Sephadex-purified material showed *in vivo* activity per unit weight 6 times that of crude material, against the Krebs-2 carcinoma tumor in mice.

DISCUSSION

This paper reports on an anticancer agent, mercenene, that is present in mollusks and is readily extractable from these tissues. Hegyeli (*15*) confirmed these findings and indicated the temperature-dependent character of the antitumor agent. Li *et al.* (*16*) demonstrated mercenene's activity in a virally induced tumor system in newborn hamsters. Judge (*17*) has found mercenene to inhibit the effects of leukemogenic viruses.

The agent studied in this report may be widely distributed in nature, especially in marine fauna. Mercenene may be ingested by the clam, a notorious concentrator, and then may be readily harvested through the extraction process. Another possibility is that some food material in this animal's environment undergoes a change *in vivo* and results in the production of a modified but active anticancer principle. Studies should be extended to include the immediate environment of the clam for a possible source of mercenene. Plankton, rich in the fauna and flora of the sea, may be the source of this antitumor agent. Other marine species should be investigated as a potential reservoir of antineoplastic agents.

In the dosages administered to achieve tumor regression and inhibition and to cause cellular oncolysis, mercenene never had a toxic effect on the general physiology of the animals. Animals of the longevity studies produced normal offspring, while the control counterparts died in 21–28 days. *In vitro* results also demonstrate a specificity of the extract for the cancer cell. Mercenene demonstrated no cytopathogenicity for the human amnion cell line FL. Antitumor agents that are nontoxic to normal cells are rare.

It has been speculated that this extract may be preventing the synthesis of DNA, RNA, or protein, and thus it is selectively killing the cancer cell. Examinations of tissue sections by light and electron microscopy have failed to reveal the mechanism whereby mercenene achieves its effectiveness (*17*). A knowledge of the cellular kinetics of the K-2 tumor is essential before we can actually determine the control mechanisms involved in the activity of oncolytic agents. The cytokinetics of K-2 have recently been reported (*18*). This study establishes the basic parameters for investigating those changes induced in the normal cell cycle by any antineoplastic agent. Studies are in progress to elucidate the regulatory mechanism involved in mercenene's effectiveness at the cellular level. Results would suggest (*20*) that the extract of the clam liver and whole clam may inhibit the initiation of DNA synthesis in the G_1 phase of the cell cycle.

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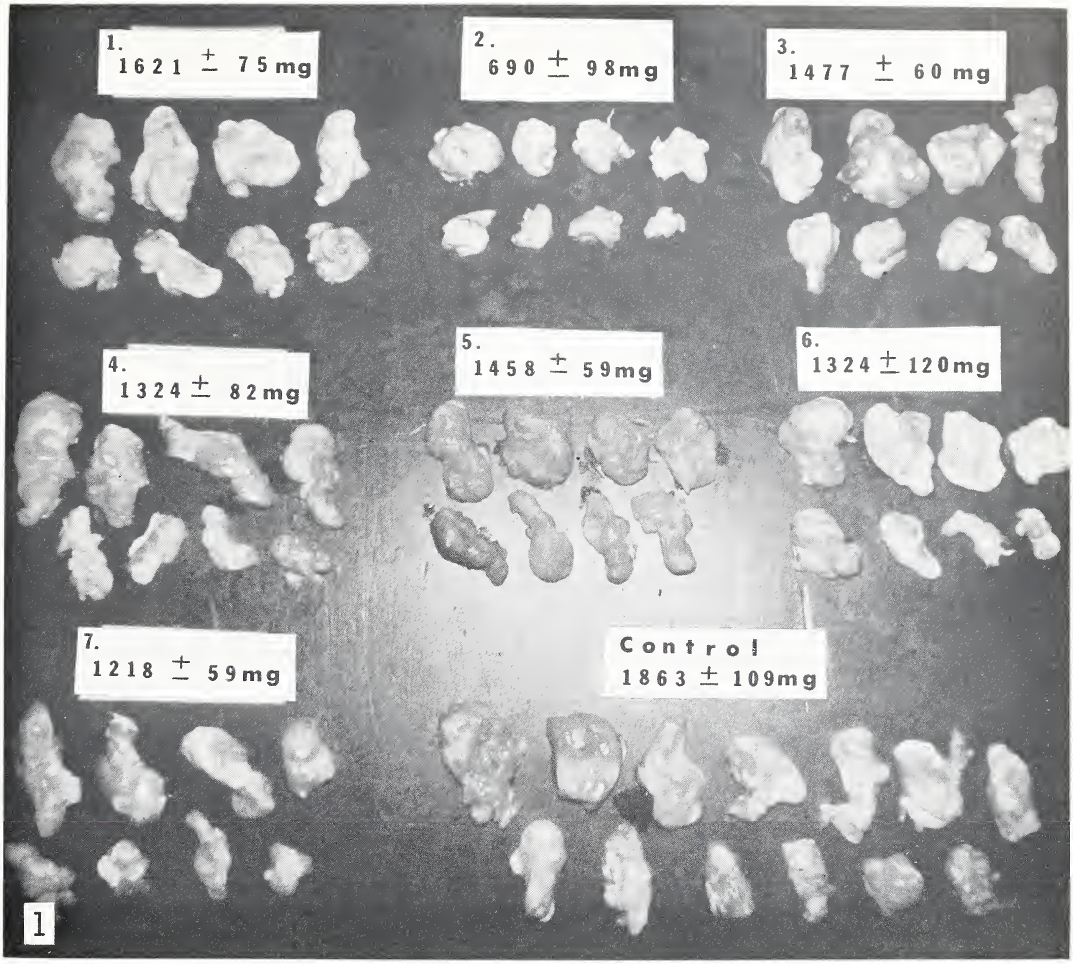
1.
1621 ± 75mg
2.
690 ± 98mg
3.
1477 ± 60 mg
4.
1324 ± 82mg
5.
1458 ± 59mg
6.
1324 ± 120mg
7.
1218 ± 59mg
Control
1863 ± 109mg
1

Figure 1.—Excised tumors from the experiment described in text-figure 1. Numbers 1–7 represent the first 7 void volumes (V_o). In each instance there was some antitumor activity due to a smearing of the activity into each void volume. However, V_o No. 2 contained the greatest concentration of activity when compared to that of the control group.

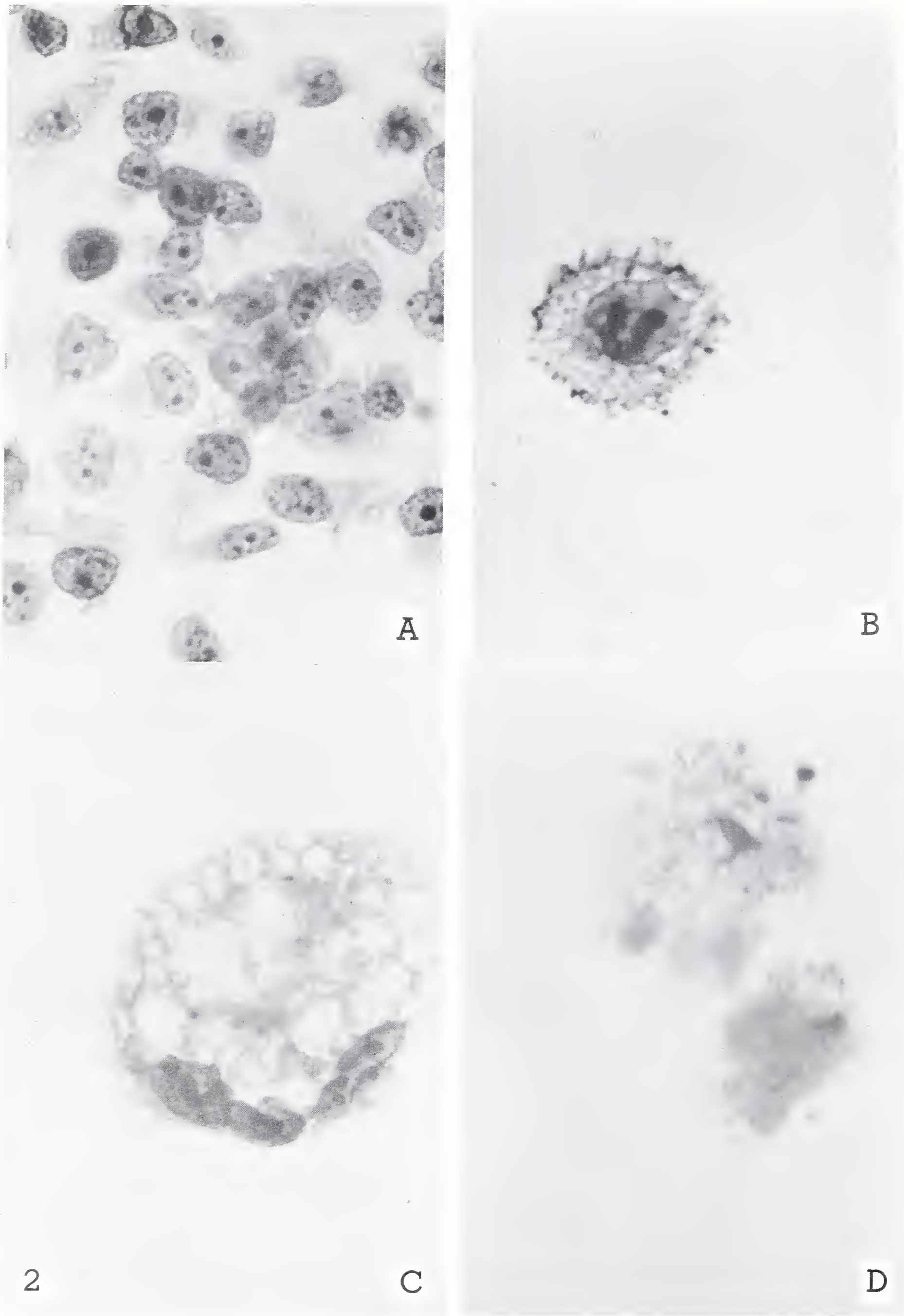

FIGURE 2.—The Syverton scale for cell degeneration of HeLa cancer cells by mercenene. *A* = An early 1+ degeneration with the cells beginning to round-up: Cytoplasmic extensions are absent in many cells; a granular appearance and vacuolization are evident. × 540. *B* = A 2+ degeneration: Membranes are thickened; there are increased granulation and extreme vacuolization. × 1200. *C* = A 3+ on the scale: Nucleus has moved to the cell's periphery; fragmentation of the cell is apparent: cell death is imminent. × 1200. *D* = A 4+ degeneration and death: Lysis. × 1200. May-Grünwald-Giemsa.

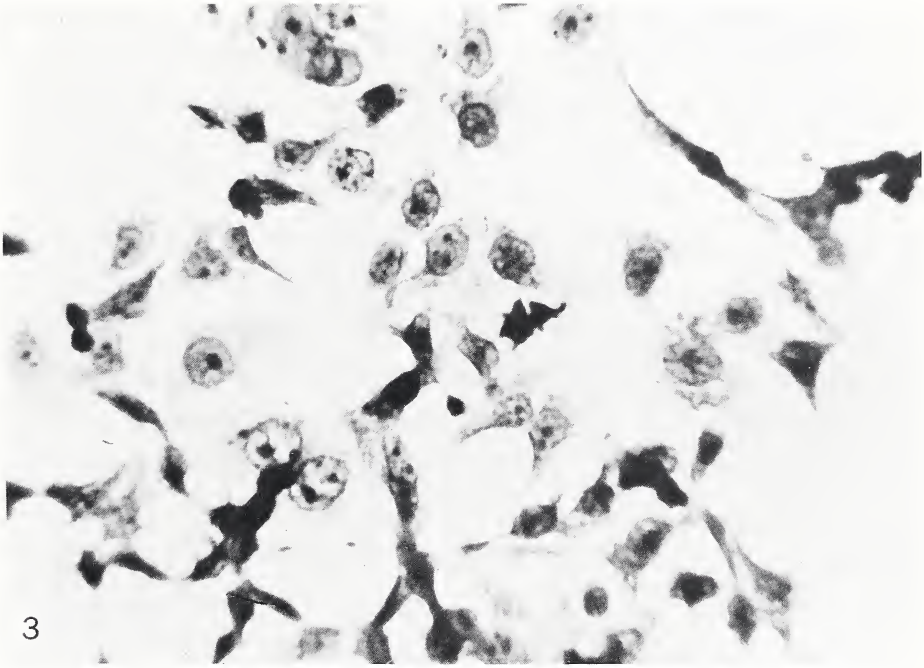

FIGURE 3.—Human amnion cells treated with mercenene in a dosage schedule identical to that described for the HeLa cells. Forty-eight hours after the addition of extract, no cytotoxicity is observed. Cytoplasmic extensions appear normal, cell membranes are not thickened, the nuclei appear normal, and no granulation or vacuolization is apparent. May-Grünwald-Giemsa. × 540

Cytological and Comparative Biochemical Studies on Normal and Neoplastic Earthworm Tissues[1]

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SUMMARY

Investigations of several neoplastic-like lesions of *Lumbricus terrestris, e.g.*, the pharyngeal tumor with giant nuclei, led to cytological studies of the nucleolar organizer. These investigations into the nature of the nucleolar organizer initiated biochemical studies on nucleic acids. Several aspects of RNA biochemistry were reviewed with the use of vertebrate and invertebrate (worm) preparations. Cell supernatant fractions from *Lumbricus* tissues were unable to methylate *Escherichia coli* sRNA. However, *in vivo* experiments showed that worm sRNA becomes methylated. Of several sources of enzymes, only mouse hepatoma sRNA methylases used worm sRNA as a substrate for the sRNA methylation reaction. *E. coli* leucyl : tRNA synthetase used worm tRNA. However, worm leucyl : tRNA synthetase could not attach leucine to *E. coli* sRNA. No 4-thiouracil was found by spectral examination in the worm, mouse hepatoma, mouse liver, yeast, rat liver, or *Bacillus subtilis* sRNAs. However, it was detected in strains B, K12, and W of *E. coli* sRNA. Although highly purified worm sRNA had an identical maximum, the worm sRNA had a bathochromic shift of about 6 mμ in the minimum when compared to the spectrum of *E. coli* sRNA. Supernatant fractions of worm tissues had high 5′ nucleotidase activity as compared with 2′, 3′ nucleotidase activities. Mouse liver preparations had low 5′ as compared with 2′, 3′ nucleotidase activities. Worm supernatant fractions had higher ribonuclease activities than mouse liver and no adenosine deaminase could be detected, though mouse liver was an excellent source. Worm sRNA had a higher melting temperature (Tm) than *E. coli* sRNA, but was less stabilized in the presence of magnesium. The nucleic acid biochemistry of worm tumor tissues remains to be explored. Studies comparing the biochemistry of tumors in invertebrates and vertebrates may give insight into the minimal requirements for the creation of a neoplastic condition.—Nat Cancer Inst Monogr 31: 593–643, 1969.

THE PHENOMENON of carcinogenesis will undoubtedly require several kinds of explanations, even if ultimately there is established only one basic mechanism. The reason for this is the nature of the cancer cell. An

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[2] I thank Mrs. P. McFarland for her excellent assistance. Many of the experiments dealing with rat enzymes were done while I was a postdoctoral fellow in the Ben May Laboratory for Cancer Research, studying under the direction of Professor H. G. Williams-Ashman.

[3] The principles of laboratory animal care as promulgated by the National Society of Medical Research are observed in this laboratory.

array of neoplastic conditions exists—some with less malignant characteristics than others, for example, the minimal deviation hepatomas. One must consider the multistates and forms of protoplasm with near-neoplastic and overt neoplastic conditions, which add to the complexity and will require explanations as to their mechanisms of inducement, development, and host relationships. Yet, protoplasm is, in many respects, similar among the various organisms, both plant and animal, and this should preclude any surprises as to certain similarities in pathological conditions. Thus the need to study tumors on a broad basis, including plant and invertebrate neoplasms, seems important to any understanding of the general phenomena of anomalous or neoplastic growths.

NEOPLASMS IN THE PHYLUM ANNELIDA [4]

One of the first reports of tumors in Annelida can be found in Needham's *Biochemistry and Morphogenesis* (*1*) and is concerned with the polychaet clam worm *Nereis* in which an ovarian sarcoma believed to be associated with degenerating ova is described. Gersch (*2*, *3*) reported on benzpyrene-induced hyperplasia in *Lumbricus.* Hancock (*4*) reported giant nuclei in pharyngeal cells of *Lumbricus*, and Stolk (*5*, *6*) (*see also* figs. 6 and 7) reported giant nuclei in *Lumbricus* associated with a pharyngeal tumor. Further cytological studies on this tumor were done by Stolk (*7*, *8*), including a histochemical study of three dehydrogenases. The pharyngeal tumors were described as having many polymorphic giant nuclei and mitotic figures. Two or more nucleoli were present in the giant nuclei, and the chromocenters were more distinct than in nuclei of normal size. Hancock (*9*) described several neoplastic-appearing lesions induced by ionizing radiation or chemical carcinogens. No attempt was made to transplant these anomalous tissues. An unusual tumor in *Lumbricus* dealing with seminal vesicle cells has been reported by Hancock (*10*) and will be dealt with in more detail in a later section. Regeneration in earthworms has been extensively studied by Gates (*11–15*).

PERTINENT HISTOLOGICAL FEATURES OF *LUMBRICUS*

A brief review of some anatomical features of *Lumbricus terristris* is presented here as a basis for interpretations of some pathological conditions presented in the next section.[5] A description of the external features of the worm includes the prostomium followed by the mouth on the ventral side and then the first somite. Other apertures include the spermathecal aperture on either side of somite X, female aperture-somite XIV, male aperture-somite XV, and the clitellum-somites XXXII to XXXVII. The dermis (figs. 30, 31, 32, and 37) is comprised of supporting cells (elongated), basel cells (club-shaped), mucous cells (large granules), albumin cells (fine granules), and sensory cells. The muscular fibers are arranged in two layers, the longitudinal and circular (fig. 17), that have pigment granules. In the fold of the intestine is the typhlosole (fig. 50) containing chloragogen cells. In the cytoplasm of the chloragogen cells are numerous globules (fig. 47), the chloragosomes, which appear to be comprised of neither fat nor albumin, but possibly chitinoid material. These cells do not have phagocytic powers, but may extract waste matter from the blood. Since glycogen has also been found in these cells, the chloragocytes may serve as nutrient storage cells.

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[4] This review includes material from a MEDLARS information search, January 1964 through December 1967.

[5] I am indebted to Stepheson's *The Oligochaeta* (Oxford Univ. Press, 1930) for much of this information.

There are several kinds of coelomic corpuscles, one being the amebocytes (fig. 81) which have many pseudopodia and can phagocytize. Lamprocytes are large cells surrounded by a pellicle and have many vacuoles containing refractile granules. The pharynx has diverticulae and columnar epithelium (fig. 3). Dorsal to the pharynx is the pharyngeal gland with polygonal and crescent-shaped cells called chromophils (figs. 1 and 2), which are not exclusive to the pharyngeal gland. Chromophils are believed to form mucin. The esophagus widens into an area called the calciferous gland (figs. 10 and 11) which is comprised of segmental dilations containing lamellae and derives its name from the reported secretions of small crystals of calcium carbonate. The thin-walled crop (fig. 17) is followed by a thick-walled gizzard (fig. 12). The most remarkable aspect of the intestine is the dorsal invaginations, termed the typhlosole, filled with chloragogen cells separated by muscle fibers at the base of the typhlosole. The function seems to be to increase the absorptive surface of the intestine. A cerebral ganglion connects by circumpharyngeal connectives to a ventral nerve cord. The nephridia (fig. 18) are comprised of the distal nephrostome (fig. 15) followed by a funnel, a narrow tube partly ciliated, and a wider, entirely ciliated tube that expands into an ampulla, finally into a muscular duct, and out to an external orifice, the nephropore.

Two pairs of testes (somites X and XI) on the ventral wall are enclosed in the male funnels (fig. 27). The testicular sacs continuing into the three pairs of seminal vesicles (fig. 19) contain groups of cells being set free by the testes. The seminal vesicles contain a fluid in which the maturation of the sperm takes place. The spermatozoa return to the testis sac and are discharged by the sperm funnels which are ciliated, passing eventually from the male aperture of somite XV. The single pair of ovaries (fig. 53) are located in somite XIII. The mature oocytes are set free into the ovarian funnels and remain in the ovisac, a dilation of the oviduct, before passing out of the female openings in somite XIV. A cocoon is formed from a secretion of the clitellum (fig. 43) that contains two types of glandular cells, one with fine and one with coarse granules. The cocoon passes over the female opening receiving ova and over the seminal receptacle (spermatheca) (fig. 13) duct opening receiving sperm that originally came from another individual during copulation. Spermatogenesis itself begins with the formation of follicles of germ cells in the testis (fig. 33). The testis contains 3–64 nuclei. By dissociation, groups of 2–8 cells are formed in the seminal vesicle and morulae (fig. 21), which are comprised of up to 128 cells formed by multiplication during maturation. Many transitional forms up to the stage of mature spermatozoa occur in the seminal vesicle. The nuclei of germ cells in the testis contain 32 small spherical chromosomes. Old morulae have amorphous masses occupying the centers which finally become vacuolated and show concretions, at last disintegrating and thus releasing the spermatozoa (fig. 25).

SEVERAL ASPECTS OF PATHOLOGY IN *LUMBRICUS*

Further observations on abnormal cells, including extensions of those published, are presented in this section. Many of these lesions were induced with saturated acetone solutions of 3-methylcholanthrene (MCA) or X irradiation. (The induction of pharyngeal giant nuclei now has been achieved with MCA, figs. 4, 5, 7, 8, and 9.) Figure 39 shows an increase in goblet cells, with bizarrely shaped hyperchromic nuclei, in a dermis after 22 daily applications of MCA (fig. 40). Other responses include areas of an increased proliferative reaction of an unknown cell type (fig. 41). The clitellum responded to MCA painting by a loss of most cell types, including the albumin cells, and a regenerative epithelial reaction after 45 days of treatment (fig. 42). Other areas had a complete loss of normal organization with subsequent replacement by a small, densely staining nucleated cell that proliferated well beyond the original borders of the worm (figs. 44 and 45). A chloragogen cell tumor (*8*) is presented more fully here. The chloragogen cells above the typhlosole continue dorsally (fig. 46) and normal chloragogen cells can be seen in figure 47. Similar cells (fig. 49) have dark-staining cytoplasm with coarse granules, but more flattened cells comprise the dorsal mass. Also several other cells appear in areas. One type is rounded but still possesses granules with the same staining features but, as in chloragogen cells, fewer of them (fig. 48).

The gonads seem to be sensitive to MCA treatment. The ovary has less ova and is replaced in part by a densely staining nucleated cell (figs. 54–56). The normal cell of the testis has a pronounced peripheral chromocenter pattern, but most testicular nuclei in worms that had been painted for 40 days with MCA had chromatin extremely condensed and ill-defined. The seminal vesicle also responds dramatically by losing all its morulae and other cellular elements (fig. 64). However, similar conditions of the seminal vesicle have been occasionally seen in untreated worms (figs. 62 and 63). An unusual tumor of the seminal vesicle area, reported previously (*9*), had a mixture of cell types in the ventral proliferating mass (figs. 65–69), including mature sperm and a completely new cell type. Hancock (*16*) has reported on irradiation-induced neoplasms in *Lumbricus*. X irradiation can create a cell type (figs. 74–77) that is quite invasive, penetrates into muscle layers, fills the coelomic cavity, and, in the case presented, proliferates through the dorsum to create great masses (figs. 71, 72, and 74–77). Multinucleated giant cells are common in the coelom in both X-irradiated specimens and MCA-treated ones (figs. 73 and 78–80). Incidentally, the lethal dose (LD50/35 days) for X ray has been determined for *L. terrestris* at 100 kr (*17*).

Several miscellaneous lesions are presented in figures 78–82. A lesion produced by acetone treatment of an induced burn area (figs. 83 and 84) demonstrates a cell common in regeneration. The hyphae of a mycotic mass published in (*9*) are shown in figure 85. In figures 86–88, an anomalous dissociated or "ascites tumor-like" cell was dispersed over large areas and caused a general disorganization of tissue component, filling the coelomic cavity of a worm that had received MCA paintings for 22 days. Similar cells could be seen in a worm treated for 30 days with MCA (fig. 89). An untreated spontaneous lesion (fig. 90) that disrupted the periphery as a white, slightly raised area could be seen microscopically to disrupt the pattern of the muscle fiber and possessed a great many bizarrely shaped cells. Figure 91 demonstrates a hyperplastic response of the dermis after 56 days of MCA treatment.

NUCLEIC ACID METABOLISM OF INVERTEBRATES AND VERTEBRATES

Soluble Ribonucleic Acid (sRNA) Methylation in an Invertebrate (*Lumbricus*) and a Vertebrate (*Mus*)

By using sRNA from a particular mutant *Escherichia coli*, Fleissner and Borek (*18*) were able to demonstrate that minor nucleotides present in sRNA, such as thymine ribose-phosphate, had not been incorporated from preformed nucleotides, but had been modified by enzymatic alkylations at the polymer level. Thus a hypomethyl or methyl-deficient sRNA acted as a substrate when L-methionine or more specifically *S*-adenosyl-L-methionine (SAM), the immediate precursor, was present in the soluble cell fraction. Many studies in Borek's laboratory (*19–21*), Hurwitz's laboratory (*22*, *23*), and in the writer's laboratory (*24–27*) have shown the complexity and profound biological implications of this sRNA methylation system. Littlefield and Dunn (*28*) found 5-methyluracil, 2-methyladenine, N^6-methyladenine, and N^6-dimethyladenine in yeast RNA. Adler *et al.* (*29*) reported N^2-methylguanine and 1-methylguanine in yeast RNA. These have been found in sRNA specifically (*30*, *31*). Hall (*32*, *33*) isolated 1-methylinosine from yeast sRNA and 3-methyluridine and 3-methylcytidine.

Holley *et al.* (*34*) found that the various specific sRNAs differed in the methylated nucleotide content. Alanyl transfer ribonucleic acid (tRNA) contains methylated guanylic acid but no methyl adenylic acid. Valyl tRNA, on the other hand, contains methyl adenylic but no methyl guanylic acid; 5-methyluridine and uridine (Ur) are found in alanyl, tyrosyl, and valyl tRNAs. Borsook and Dubnoff (*35*) showed that adenosine triphosphate (ATP) was required for enzymatic methionine transmethylation reactions. Cantoni (*36*) isolated the enzyme from rabbit liver for the synthesis of active methionine and also characterized this intermediate (*37*) as SAM having the methionine sulfur bonded at 5′ position.

If methyl nucleosides or bases are given to organisms, they are not incorporated. N^6-methyladenine, for instance, will not incorporate into sRNA, but did appear in the RNA after demethylamination (*38*). RNA polymerase *in vitro* will incorporate 5-methyluridine triphosphate. A given source of RNA will act as if it is fully methylated if its homologous methylase is used, but new methylation occurs if a different species of methylase is used. Prior irradiation of DNA appears to allow supernumerary methyl groups to be established by methylase enzymes (*20*). Methylase is found in pea-seedling nucleoli but also in nuclei and cytoplasm of animal cells (*19*). Ribosomal RNA (rRNA) methylases are believed to be separated from the enzymes methylating tRNAs (*39*).

Borek (*21*) has theorized on the functional significance of the methylated nucleotides in tRNA. There appeared to be no increased degradation of nonmethylated tRNA when pancreatic ribonuclease was used. The positions of methyl group 1, 2, and 6 on adenine and 1 and 2 on guanine and the dimethyl functions on the amino functions may contribute to weakening hydrogen bonding and maintain the loop or bend, single strand. The 5-methyluracil and the 2′ OH groups are sterically incompatible in a helical model. The ability of amino acids to become incorporated into methyl "poor" and normal sRNAs was the same (*40*); however, very recently Shugart *et al.* (*41*) have presented evidence to the contrary and show a functional relation between the extent of methylation and the extent of amino acid acylation for several amino acid acceptor activities of mixed tRNAs, and the amino acid itself is not related to the specificity of amino acid transfer reactions (*42*). Gold and Hurwitz (*43*) and Gold *et al.* (*22*) have described separate enzymes which methylate RNA and DNA.

In the experiments described below, worm supernatant cell fractions were used. Worms (*L. terristris*) were purchased from the General Biological Supply House, Chicago, Illinois. Table 1 describes attempts to demonstrate sRNA methylase activity of 100,000 × *g* supernatant fractions from whole-worm homogenates. Under a variety of conditions (*E. coli*, yeast, rabbit liver, with and without the addition of 30 μmoles of magnesium) no incorporation could be detected. A more comparable system than liver to compare whole-worm supernatant fractions may be mouse muscle. Although 1010 cpm is incorporated per 100 mg protein equivalents of supernatant with mouse liver cells, only 54 cpm per 100 mg protein (considered only trace activity) occurs with mouse skeletal muscle preparations, and 138 cpm per 100 mg protein for whole-worm supernatants. Worm sRNA was prepared by the Kirby phenol method (*44*), which had a 280/260 ratio of 0.585.

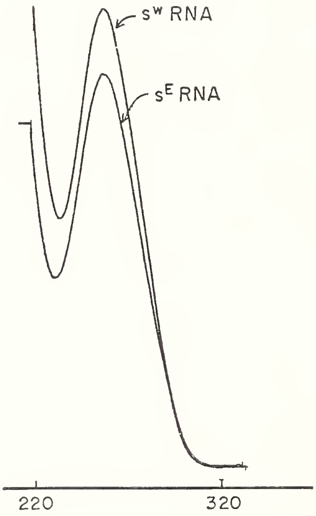
In text-figure 1 the spectrum of worm sRNA is compared with commercial *E. coli* sRNA. The spectral qualities of the worm sRNA prepara-

TABLE 1.—Comparison of high-speed supernatant fractions of mouse liver and whole worm to methylate *E. coli* sRNA*

Enzyme fraction	*E. coli* sRNA	cpm incorporated
Dialyzed worm supernatant	−	104, 115
	+	123, 89
Undialyzed worm supernatant	−	89
	+	80
Dialyzed mouse liver supernatant	−	201
	+	580

*Reaction mixtures contained 100 μmoles Tris, *p*H 7.6, 50 μmoles glutathione, 30 μmoles $MgCl_2$, 100 μg of *E. coli* K12 sRNA as indicated, 0.5 μc of *S*-adenosyl-L-(^{14}C-methyl)-L-methionine (47.5 mc/mmole), and 1 ml of undialyzed 100,000 × *g* worm supernatant fraction (27.2 mg protein/ml), dialyzed worm supernatant fraction (24.8 mg protein/ml), or dialyzed mouse liver supernatant fraction (20.7 mg protein/ml) in a total volume of 2 ml; incubation was for 1 hour at 37°C. The assay method of Srinivassan and Borek (*19*) was used.

tion are good. Note that the minimum of the worm sRNA absorption spectrum has a bathochromic shift, though the maxima of *E. coli* and worm sRNA are identical. The significance of this is not clear but may be due to a difference in the amount of a particular nucleotide. The results of many studies using worm sRNA showed no activity with yeast, *E. coli*, mouse liver, or *Lumbricus* enzyme fractions. Deoxyribonuclease pretreatment of the sRNA did not allow incorporations. However, a preliminary experiment using BW7756 mouse hepatoma sRNA methylase activity on worm sRNA showed a definite incorporation of methyl groups onto the polymer. Therefore, the experiment in text-figure 2 was devised which clearly shows methylation of worm sRNA by the neoplastic cell enzymes. With the curious inertness of worm sRNA it was decided to investigate the ability of L-methionine, *S*-adenosyl-L-methionine (SAM), and formate (a control for a source of one carbon) for one-carbon utilization for sRNA synthesis—presumably as the methyl group. The results are shown in table 2. If the pool concentration of the precursor is not considered, it appears that formate was used 57% as well as SAM for methylation and L-methionine appeared to be 187% as effective as SAM. However, if one takes into consideration the original pool utilized, one finds that SAM had one-half the pool concentration of formate, yet it was twice as effectively used for methylation of sRNA and had about one-twentieth the concentration of L-methionine, yet SAM use was still 50% of that shown by L-methionine. Apparently, L-methionine is used, but not as well as formate, in sRNA synthesis. Therefore it would be difficult to decide by this evidence if the terminal methyl group of L-methionine is used directly (via SAM) for methylation of sRNA or if it is used via the one carbon pool for synthesis of the base moiety. However, of the three precursors, SAM is used best and is an immediate precursor of sRNA synthesis—namely methylation. Therefore, the L-methionine data probably reflect its use as a precursor of SAM synthesis and very little donation via the one-carbon pool *per se*. Previous calculations on the rate of *in vitro* methylation in mouse liver (*24*) gave 168 $\mu\mu$moles of methyl groups incorporating into 0.75 mg of mouse liver sRNA per hour, and similar values were calculated theoretically for *in vivo* conditions. Data available on the pool size of SAM, 77 mμmoles, indicated that mouse liver uses about 0.23% of the SAM pool for sRNA methylation. From the above it was determined that 223 μμmoles of methyl groups is incorporated per hour per mg sRNA in mouse liver *in vitro*. Therefore, the order of magnitude for *in vitro* worm methylation agrees with the *in vitro* methylation of mouse liver sRNA. Furthermore, it would be expected that the rate of sRNA methylation for a poikilotherm would be lower than that of a homiotherm. The methyl group from SAM is covalently bonded to nucleotides (text-fig. 3). The sRNA was isolated from worms given injections of labeled SAM and then subjected to alkaline hydrolysis of the diester bonds before resolution on high-voltage paper electrophoresis. Some sRNA apparently did not become hydrolyzed and remained near the origin. Two other fast-moving methylated components have not been identified. SAM and L-methionine migrate in the opposite direction as do nucleosides and bases under these electrophoretic conditions.

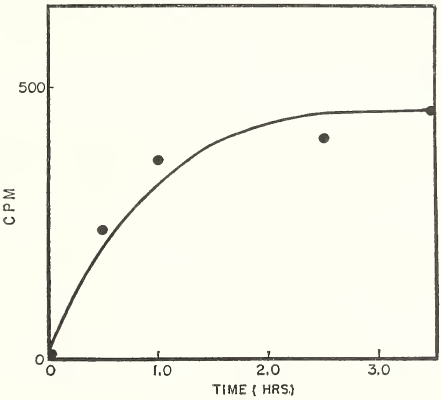


TEXT-FIGURE 1.—The absorption spectrum (220–320 mμ of *Lumbricus* (*3*) and *E. coli* (E) sRNA.

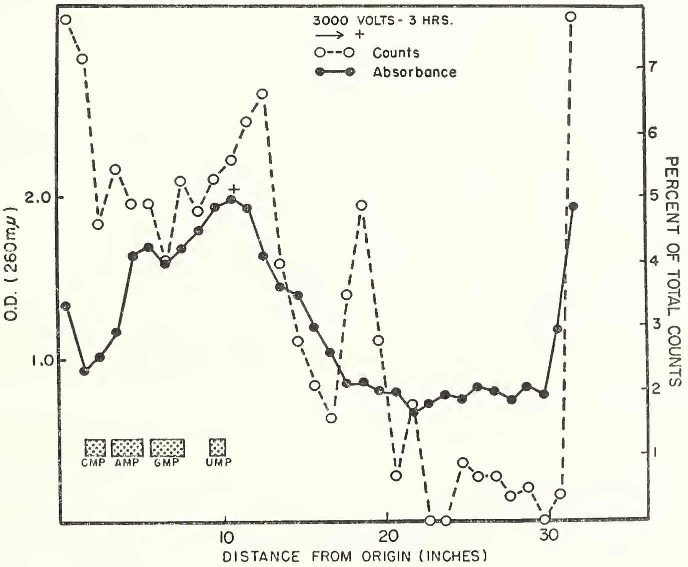
Other Biological and Physical Properties of Worm sRNA

Thiouracil analysis.—Lipsett (*45*) has described a 4-thiouracil minor nucleotide of sRNA and its spectral properties. An examination of a series of sRNAs from commercial sources (except for mouse liver, hepatoma, and worm sRNA) (table 3) showed that only *E. coli* sRNA strains had a 328–336 mμ absorption peak. Therefore, no evidence for 4-thiouracil has been found in worm sRNA.

Tm studies.—The hydrogen bonding and secondary structure of an RNA molecule can be studied by thermal denaturation of the helical content which increases the aromatic character of bases and increases the 260 mμ absorption. The secondary structure of worm sRNA was compared with *E. coli* sRNA (text-fig. 4). The Tm is a particular temperature at which one-half the total hyperchromicity has developed. The Tm of worm sRNA is 55°C or about 7° higher than *E. coli* sRNA, suggesting more GC content



TEXT-FIGURE 2.—Methylation of *Lumbricus* sRNA by mouse hepatoma (BW7756) sRNA methylases. Enzyme reaction mixture contained 600 μmoles Tris buffer, *p*H 7.6, 180 μmoles MgCl$_2$, 300 μmoles reduced glutathion, 1.2 mg *Lumbricus* sRNA, 3 μc *S*-adenosyl-L-(^{14}CH$_3$)-methionine iodide (50.5 mc/mmole), and 6 ml of mouse hepatoma (BW7756) 100,000 × *g* supernatant fraction (22.1 mg protein/ml), in a total volume of 12 ml; incubation was at 37°C; 2 ml samples were removed at 0, 0.5, 1, 2.5, and 3.5 hours.



TEXT-FIGURE 3.—High voltage electrophoresis of alkaline hydrolysate of sRNA from worms treated with *S*-adenosyl-L-(^{14}CH$_3$)-methionine.

TABLE 2.—*In vivo* utilization of one-carbon sources for sRNA synthesis in *Lumbricus**

Precursor	μμmoles incorporation/mg worm sRNA/hr	Pool concentration of precursor (mμmoles/100 g wet weight tissue)
Formate	46. 2	2. 28
L-methionine	168. 0	23. 10
S-adenosyl-L-methionine	89. 3	1. 09

*Three lots of worms of 100 g each were rinsed and inoculated with approximately 10 μc *S*-adenosyl-L-(^{14}C-methyl)-L-methionine (50.2 mc/mmole), iodine ^{14}C-formate (113 mc/mmole), or ^{14}C-methyl-L-methionine (10.3 mc/mole) and killed 1 hour later, homogenized in 1 volume of 0.25 M sucrose —0.01 M Mg; 10,000 × *g* and subsequent 100,000 × *g* supernatants were prepared. Phenol method of Kirby (*44*) was used for the isolation of sRNA.

TABLE 3.—4-Thiouracil content of sRNA by spectral examination

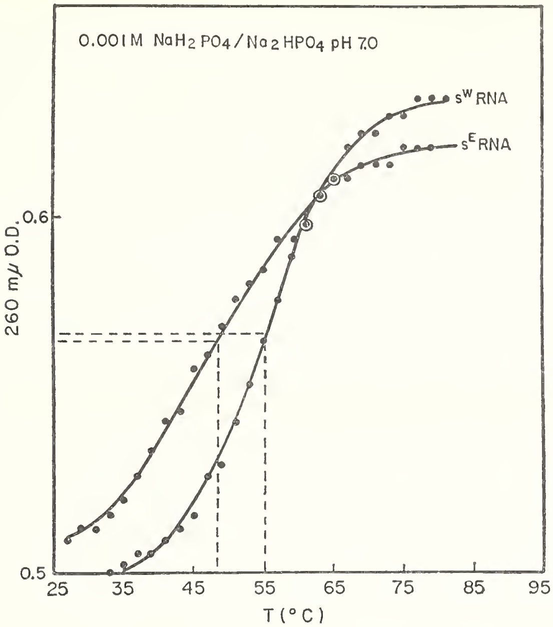
sRNA	Percent of 260 mμ absorption	Maximum absorption (mμ)
Yeast (nonstripped or stripped)	—	None
B. subtilis	—	None
Rat liver	—	None
E. coli strain B	1. 64	(332)
E. coli K12	1. 67	(336)
E. coli strain W	2. 20	(328)
Mouse liver (C57BL/Ks)	—	None
Hepatoma (BW7756)	—	None
Worm (*Lumbricus*)	—	None

of the average sRNA or that specific tRNAs with higher GC content are more plentiful. The amount of secondary structure of worm sRNA is similar to *E. coli* sRNA. In the presence of 1 mM magnesium (text-fig. 5), the stabilizing effect shown by Monier and Grunberg-Manago (*46*) for yeast sRNA is seen for worm sRNA, since the Tm was shifted 16°s. However, the magnesium did not stabilize the secondary structure as much as with *E. coli* sRNA.

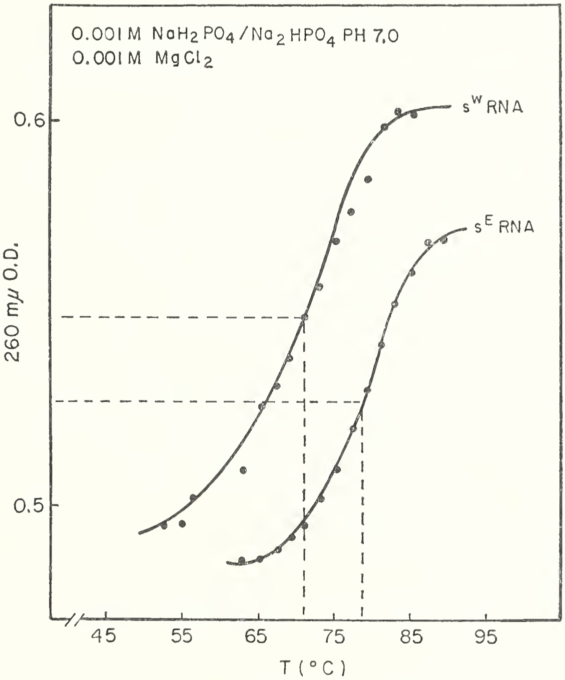
Amino acid acceptor activity.—Table 4 presents data showing that worm leucyl tRNA is an excellent acceptor of leucine by both *E. coli* and worm supernatant fractions. However, worm leucyl-tRNA synthetase did not add leucine to *E. coli* leucyl tRNA, although *E. coli* supernatants were active in this respect. Such species specificity of amino acid: tRNA synthetases has been reviewed by Brown (*47*).

RNA Catabolism Studies With Invertebrate and Vertebrate Cell Fractions

Ribonuclease activity of rat prostate, mouse liver, and worm cell fractions.—Phosphodiesterase activity can be divided into those attacking polyribonucleotides and those attacking nucleoside cyclic monophosphates. A particularly active enzyme which hydrolyses poly U (orthophosphoric diester phosphohydrolase E.C. 3.1.4.1) was found in the supernatant fluid after ultracentrifugation of rat prostate homogenates.



TEXT-FIGURE 4.—Absorbance-temperature profiles of *Lumbricus* (w) and *E. coli* (E) sRNA without magnesium.



TEXT-FIGURE 5.—Absorbance-temperature profiles of *Lumbricus* (w) and *E. coli* (E) sRNA with magnesium.

TABLE 4.—Leucine acceptor activity

Enzyme	sRNA (cpm/mg)	
	E. coli	Worm
E. coli	355	1402
Worm	0	1842

*The reaction mixture contained 100 μmoles Tris, *p*H 7.6, 5 μmoles $MgCl_2$, 10 μmoles KCl, 4 μmoles glutathione, 10 μmoles ATP, 9 μmoles CTP, and either 400 μg of *E. coli* sRNA or 265 μg worm sRNA, 6 μc ^{14}C-leucine (71 mc/mmole), 0.2 ml *E. coli* supernatant fraction (14.9 mg protein/ml), or 0.2 ml worm supernatant fraction (18.5 mg/ml) prepared by the method of Cantoni and Richards, in a total volume of 1 ml; incubation was for 15 minutes at 37° C. Appropriate blanks containing no sRNA are included. The reaction was stopped after the addition of 1 mg of yeast RNA and 6 ml of 0.5 M NaCl in 67% ethanol. The mixture was allowed to stand for 20 minutes and centrifuged at 14,000 × *g* for 10 minutes. Precipitates were washed 3 times with NaCl-ethanol, finally suspended in 1 ml of 1.5 M NH_4OH, placed into planchettes, and counted.

Methods.—Polyuridylic acid (poly U) was prepared by the action of the polynucleotide phosphorylase of *Micrococcus lysodeikticus* on UDP. ^{14}C-labeled poly U was prepared with UDP-2-^{14}C as starting material. The polynucleotides were incubated with 10,000 × *g* extracts of rat prostate for various times at 37°C, and the reactions were terminated with ethanol or 1 M HCl-ethanol to give a final concentration of ethanol of 80% by volume. After standing for 1 hour at 4°C, the mixtures were centrifuged at 14,000 × *g* for 30 minutes. Material made acid-soluble was determined by measurement of radioactivity or of absorbance at 260 mμ. Suitable controls were run to correct for any radioactive or UV-absorbing material in zero-time samples.

The typical substrate was made with a polynucleotide phosphorylase enzyme isolated by the method of Steiner and Beers (*48*) from *M. lysodeikticus* using UDP-^{14}C.

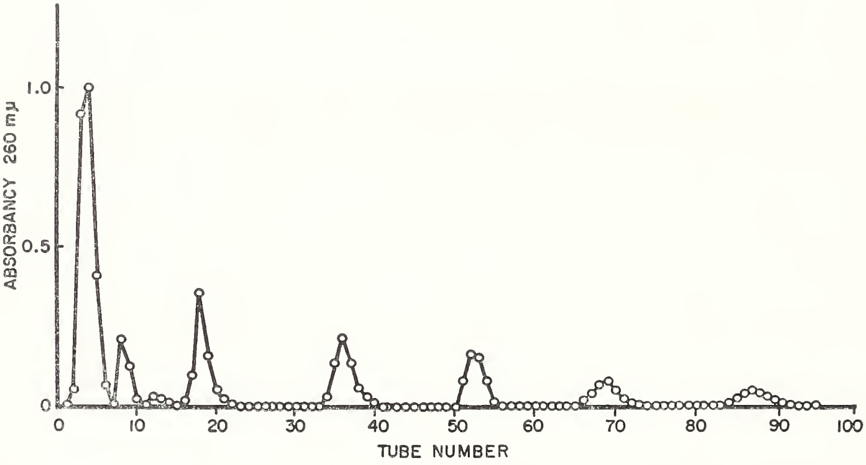
Forty g of *M. lysodeikticus* powder was autolyzed and then subjected to a 40–50% ammonium sulfate precipitation. The reaction system contained: 4.32 mg protein enzyme, 1 mmole Tris, *p*H 9, 25 μmoles Mg, and 100 μmoles UDP containing 1 μc of 12.5 μc/mg of 2-^{14}CLi_3 UDP in 10 ml total volume; incubation was for 60 minutes, and assaying for P_1 release was done at varying intervals. The reaction mixture was adjusted to *p*H 8 with 1 M HAc, and stopped with 3 volumes ethanol; 0.2 ml of 1 M NaCl was added; the mixture was centrifuged and decanted, and then 5 ml of NaCl-citrate solution was added; the mixture was stirred, and 5 ml of saturated aqueous phenol was added with stirring for 20 minutes at 29°C; reextracted with 1 ml of water, to the combined aqueous layers ether was added, and was washed until clear and dialyzed against 0.02 M KCl. The final product had a specific activity of 1740 cpm/100 μg.

A sucrose gradient analysis of ^{14}C-poly U was done by placing a sample on 4 ml of 5–20% sucrose gradient, centrifuging in an SW 39 head at 38,000 rpm for 6 hours, collecting 10 drop samples, and measuring 260 mμ absorption. The gradient analysis showed about 80% of the product to be of approximately 3 S weight and 20% a heavier type (approximately 4–10 S).

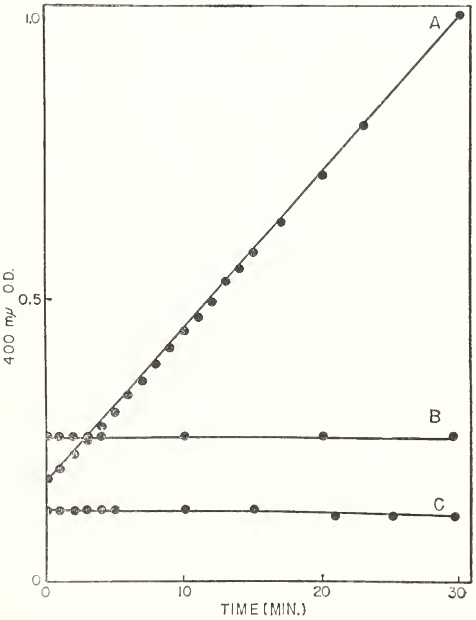
Copolymers were made by using ADP and UDP with this same enzyme method. Other substrates used from commercial sources were yeast RNA, yeast sRNA, *E. coli* rRNA, and turnip yellow mosaic virus RNA (gifts from Dr. R. Haselkorn), calf thymus DNA, "core" RNA, poly UMP, AMP, CMP, 2:3 cyclic UMP and 3:5 cyclic AMP, and *bis*(*p*-nitro phenyl) phosphate; nucleotides were used for synthesis of polymers. Chromatography was done using the methods of Pabst II system (*49*). Oligonucleotides were chromatographed by the method of Tener *et al.* (*50*), on diethylaminoethyl (DEAE) cellulose, and sRNA chromatography with the method of Sueoka and Cheng (*51*) using celite and methylated albumin. Amino acceptor activity of sRNA was done with ^{14}C-phenylalanine. The prostate enzyme fractions were made from tissues of mature male Sprague-Dawley rats.

Results.—Rapid conversion of poly U to produce soluble products in acid and 80% ethanol was found to be catalyzed by the supernatant fluids resulting from centrifugation for 1 hour at 105,000 × *g* at 4°C, or in rat ventral prostate homogenates prepared according to Liao and Williams-Ashman (*52*). Such extracts cleaved approximately 0.1 μmoles of uridylic acid per mg of protein per hour at 37°C. Most of the poly U-degrading activity was precipitated by ammonium sulfate at 0°C between 20 and 60% saturation; the fraction precipitating between 20 and 40% saturation exhibited the highest specific activity. The optimum *p*H of the reaction was approximately 8. At a final concentration of 1 mM, neither magnesium chloride nor potassium phosphate affected the rate of poly U breakdown. The formation of ethanol-soluble breakdown products from poly U was proportional to the time of incubation and to the amount of enzyme added, provided not more than 30% of the poly U, initially added, became degraded and the enzyme concentration was not greater than 3 mg of protein per ml of reaction mixture. Poly U degraded to a series of oligonucleotides as ascertained by using a DEAE cellulose column by the method of Tener *et al.* (*50*). Seven well-resolved peaks believed to represent mono- through hepta-oligonucleotides were found by this method (text-fig. 6). By the use of a DEAE cellulose column and gradient elution (0.01–0.3 M phosphate buffer 7.0), the 5′ nucleotidase activity present in the 20–60% ammonium sulfate fraction was resolved from the diesterase activity. When the purified 5′ nucleotidase was examined for its effect on poly, the mononucleotide product was found to be 5′-uridylic acid (UMP). Polyribonucleotide degradation was also demonstrated by crude supernatant preparations from prostate tissue of C57BL/6J mice.

The writer has investigated several aspects of sRNA structure and function with the use of a diesterase derived from prostate tissue. The diesterase also attacked an organic diester (text-fig. 7), and experiments designed to relate its activity to the amount of secondary structure displayed by the substrate gave a good correlation (*see* table 5). The greater the percentage of secondary structure possessed by a given polyribonucleotide, the smaller was the degradation. Investigations as to the effect of the protein fraction from 20–40% ammonium sulfate on the sRNA molecule were done by the use of ^{14}C-phenylalanine, assaying the acceptor ability of the sRNA (text-fig. 8). sRNA treatment was done using a reaction system containing *E. coli* sRNA, enzyme preparation, and buffer; incubation was for 3 hours at 37°C. the sRNA was reisolated by a phenol treatment and after dialysis was then assayed by being placed into a system containing ^{14}C-phenylalanine (12.5 μc specific activity, 140 mc/mmole), buffer *p*H 7.5, ATP, phosphoenolypruvic acid, Mg^{++}, pyruvic kinase, cytidine triphosphate (CTP), and *E. coli* *p*H 5 supernatant.

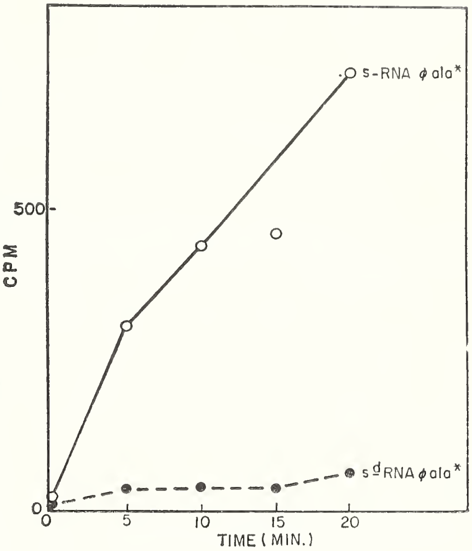


TEXT-FIGURE 6.—Resolution of mono- through hepta-oligonucleotides of uridylic acid. Sample of acid-soluble enzyme reaction mixture, containing polyuridylic acid after rat prostate ribonuclease treatment, was placed onto a DEAE cellulose column, and a gradient elution of 0–0.4 M LiCl in 0.04 M lithium acetate,*p*H 5, was used.



TEXT-FIGURE 7.—Diesterase activity of ammonium sulfate fraction of rat prostate. The reaction system (A) contained 5 μmoles *bis* (paranitrophenyl) phosphate, 100 μmoles of Tris, *p*H 8.2, and 750 μg of 20–40 enzyme in a total volume of 2.05 ml; incubation was at 31°C during spectral analysis. Controls were made by using no enzyme (C) and by using a heat-denatured (90° for 30 min) enzyme preparation (B).

Text-figure 8.—The ability of *E. coli* sRNA treated with rat prostate ribonuclease to accept amino acids. Incubation mixture contained 500 μg of sRNA (untreated *E. coli* sRNA) or 500 μg of s^{d}RNA (*E. coli* sRNA which had been treated in the following manner: 10 mg of *E. coli* sRNA, 15 mg protein of 20–40% ammonium sulfate fraction of 100,000×*g* supernatant of rat prostate tissue, 100 μmoles of Tris, *p*H 8.2, in a total volume of 3 ml were incubated for 3 hours at 37°C. The sRNA was reisolated by a phenol treatment and then dialyzed against 1 liter of water at 4°C for 48 hours. The 380/260 mμ ratio was 0.56. ^{14}C-phenylalanine (total of 0.25 μc at 140 mc/mmole), 1 mmole of Tris, *p*H 7.5, 4 μmoles ATP, 10 μmoles of phospho-enol-pyruvic acid, 5 μmoles of magnesium acetate, 100 μg of pyruvic acid kinase, 0.1 μmoles CTP, 0.02 ml of *E. coli* "*p*H 5 supernatant fraction" in a total volume of 1 ml; 0.2 ml samples were removed at various intervals and 5 ml of 5% tricholoroacetic acid (TCA), 500 μg yeast RNA, and 500 μg albumin were added. After two 5% TCA washes, one 10% TCA wash, and two ethanol-ether (3:1) washes, the precipitate was solubilized and counted in planchettes.



Samples were removed at varying intervals, and TCA, carrier yeast RNA, and albumin were added. After several acid and ethanol-ether washings, they were analyzed for radioactivity. A marked depression of the aminoacyl AMP bond formation of the terminal AMP moiety was found. Such acceptor studies have been made on sRNA using venom diesterase (*53*). Similar effects have also been reported for spleen phosphodiesterase (*54*).

Chromatographic studies were used on diesterase-treated sRNA to attempt to understand the nature of the product. When spotted on electrophoresis, a complex of spots occurred, all of which migrated slower than 2′- or 3′-UMP, except one small spot that migrated faster than UMP. Therefore, it was interpreted that most of the UMP was in the form of oligonucleotides. A sample of diesterase-treated yeast sRNA was analyzed on a DEAE-cellulose column eluted with an $LiCl_{12}$ buffered gradient. Five peaks were resolved, indicating various sizes of oligonucleotides, but when analyzed on a methylated keisulfur column (*51*), virtually all the

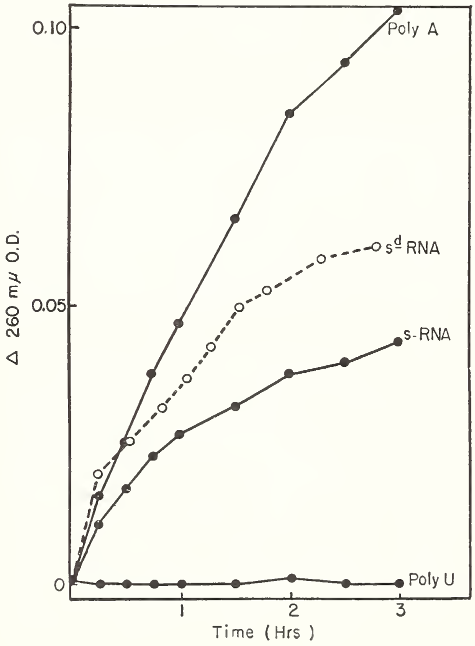
TABLE 5.—Ribonuclease activity versus quantity of secondary structure*

Polymer	Approximate amount of secondary structure (%)	Relative optical density (hr) 0	1	2	3
Poly U	0	0.069	0.314	0.610	0.710
TYMV RNA	<40	.031	.092	.171	.270
E. coli rRNA	~40	.043	.069	.110	.161
Yeast sRNA	>40	.065	.066	.091	.103

*Reaction system contained 500 μg poly U, turnip-yellow mosaic virus (TYMV) RNA, *E. coli* rRNA, or yeast sRNA, 10 μmoles of Tris, pH 8.2, and 1.5 mg of 20–40 enzyme in a total volume of 0.75 ml; incubation was at 37°C; 0.15 ml samples were removed at 1, 2, and 3 hours; 3 ml of ethanol-1 N HCl solution was added. After the mixture stood for 1 hour, it was centrifuged for 20 minutes at 14,000 × *g*, and 260 mμ absorption of supernatant was measured.

untreated sRNA eluted off as a peak at 0.4 M NaCl, but all the treated sRNA came off at 0.05 M NaCl, probably indicating a change in the size and molecular configuration, or both, of the sRNA molecule.

Thus it appeared that some changes including probably the destruction of the CCA terminal nucleotide end had occurred. Samples of yeast sRNA, diesterase-treated and untreated, were placed on a 5–20% sucrose gradient and spun at 38,000 rpm for 19 hours. A variety of changes in the molecular weight had occurred. A further study was made, based on the work of Zubay and Marciello (*55*), which utilized the concept of formaldehyde forming a Schiff base with any available amino group from adenine, guanine, or cytosine moieties and thereby indicating the extent of secondary structure present. This experiment (*see* text-fig. 9) shows an increase in available amino functions and a concomitant loss of secondary



TEXT-FIGURE 9.—Indication of changes in the secondary structure of prostate RNA as treated yeast sRNA by the formaldehyde reaction. Reaction system contained 100 μg of yeast sRNA or yeast sRNA treated as described in text-figure 8 or 25 μg of poly U acid or poly A.

structure after diesterase enzymatic treatment. Poly U and polyadenylic acid (poly A) were used as controls. Poly U gave no change in 260 mμ absorption readings as one would expect, while poly A gave a very large change.

Table 6 shows that worm supernatant fractions 100,000 × *g* are quite active in the hydrolysis of polyuridylic acid compared to the mouse liver soluble cell fraction, and, of course, commercial purified pancreatic ribonuclease activity shows a high specific activity and is used in this experiment as a control.

TABLE 6.—Ribonuclease activity of worm, liver, and beef pancreas preparations

Enzyme	Comparable 260 mμ optical density readings of final assay supernatant determined with appropriate dilutions	μmoles* UMP/hr/mg protein (mono- and oligonucleotides)
Worm supernatant fraction	1. 12	1. 50
Liver supernatant fraction	0. 14	0. 14
Pancreatic ribonuclease (crystalline)	54. 6	552. 00

*Note: 9.9 mM extinction coefficient used.

Nucleotidase and Adenosine Deaminase Studies With Rat Prostrate, Mouse Liver, Mouse Spleen, Mouse Hepatoma, and Worm Preparations

A 5′ nucleotidase has been partially isolated from prostate nuclear and microsomal preparations. Specificity studies show it to be consistent with 5′ nucleotidases derived from other sources in that it attacks all the 5′ nucleotides and degrades poorly the 2′, 3′ nucleotides or other phosphate sugars or phosphodiester linkages. The ventral prostate also contains an enzyme capable of deaminating adenosine to inosine but not cytidine to uridine. Inosine was rapidly degraded to hypoxanthine via an uncharacterized reaction.

Reis (*56*), who discovered 5′ nucleotidase, reported on its presence in human prostate. The enzyme was shown to hydrolyze phosphorus from 3′-guanylic acid (GMP), 5′-inosinic acid (IMP), 3′-AMP, and 5′-AMP, but a nucleotidase from testis was much more specific, working on 5′-IMP, 5′-AMP, and to a much lesser extent on the 3′ nucleotides. Enzymes from both sources attacked poorly, or not at all, the following substrates: glycerol phosphate (α or *6*), hexosemonophosphate, and phosphoglyceric acid.

Heppel (*57*) states that only a few 5′ nucleotidases have been characterized as to their specificity, since most work involved a single 5′ nucleotide. He further states ". . . that 5′ nucleotidases, in general, show activity against ADP and ATP. It has not been clearly established whether this is due to the same enzyme or to a contaminating activity." The 5′ nucleotidases that have been well characterized as to substrate specificity are snake venom, bull semen, bacterial, potato, and microsomal enzymes.

Bull semen 5′ nucleotidase has been shown to attack 5′-AMP, 5′-IMP, 5′-UMP, 5′-CMP, nicotinamide mononucleotide, and ribose-5′-phosphate, the latter at a much slower rate. No activity on 2′ or 3′ mononucleotides or other sugar phosphate, including riboflavin phosphate, was demonstrable (*58*). The bacterial enzyme attacks 5′-AMP, 5′-CMP, 5′-IMP, 5′-UMP, 5′-ADP, 5′-ATP, and DPN. No attack on 3′-AMP, 2′-AMP, 2′-UMP, 3′-UMP, ribose-5′-phosphate, phosphoglyceric acid, nicotinamide adenine dinucleotide phosphate (NADP), glucose-5-phosphate, UDPG, or pyrophosphate was found (*59*). The potato enzyme attacks 2′,5′-diphosphate, leaving 2′-AMP which is not the case for any other 5′ nucleotidase. The microsomal enzyme attacks 5′-AMP, 5′-IMP, 5′-CMP, 5′-UMP, and 5′-AMP. Adenine deoxyribose-5′-phosphate and riboflavin phosphate are degraded at a slow rate. No 2′ or 3′ isomers are attacked (*60*).

Another enzyme found in testis in large quantities is adenosine deaminase (*61*). This deaminase is specific for adenosine or adenine deoxyribonucleoside. Adenine deaminases are not found in most vertebrates but are found in extracts from invertebrates. They are at the same time apparently unable to deaminate adenosine or AMP (*62*). Table 7 clearly verifies the lack of deamination of adenosine by *Lumbricus* extracts, whereas mouse liver and spleen (text-fig. 10) and hepatoma tissues are the nucleotidase activity.

Studies were also done to ascertain the presence of both these enzymes in rat ventral prostate tissue and to characterize the substrate specificity of the nucleotidase activity.

Preparation of the Enzyme Fractions

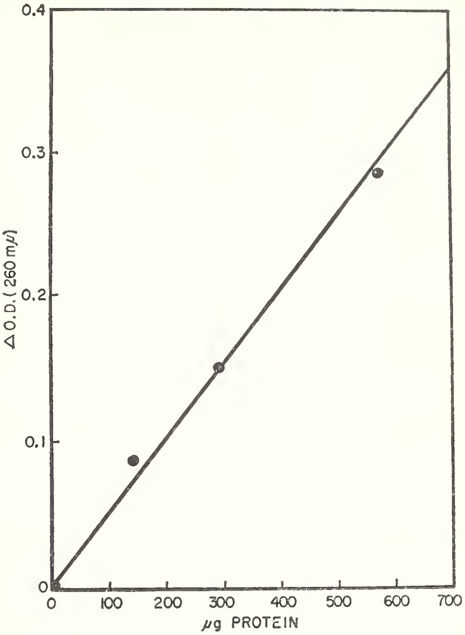
Ten grams of rat prostate tissue was homogenized in 2 volumes of a solution containing 0.01 M sucrose, 0.01 M Tris (tris-hydroxy-methyl-aminomethane HCl) buffer (*p*H 7.5), 0.001 M $MgCl_2$ for 40 strokes, filtered through 2 layers of gauze, and centrifuged at 500 × *g* for 5 minutes. The precipitate was resuspended in 10 ml of buffer and recentrifuged. This procedure was repeated before final suspension in 0.01 M Tris (*p*H 7.5).

TABLE 7.—Adenosine deaminase activity of worm, mouse liver, and hepatoma-soluble cell fractions*

Tissue	Δ 260 mμ optical density/10 min/mg protein
Worm (*Lumbricus*)	0
Mouse liver (C57L/J)	0. 204
Hepatoma (BW7756)	0. 081

*Reaction mixture contained 1.7 ml of 1.0 260 mμ optical density units of adenosine, 20 μmoles phosphate buffer, *p*H 7.0, 734 μg protein equivalents of 100,000 × g C57L/J mouse liver supernatant, or 740 μg protein of worm supernatant or 826 μg hepatoma supernatant, in a total volume of 2 ml, and was studied spectrally at 27°C.

TEXT-FIGURE 10.—Adenosine deaminase activity of C57L/J mouse spleen. Reaction mixture contained 1 ml of adenosine (1.2–260 mμ optical density units per ml), 100 μmoles phosphate, *p*H 7.0, and varying quantities of 20,000 × *g* supernatant fraction of spleen as indicated by equivalent amounts of protein.



The suspension was vibrated for 2-minute intervals 5 times, allowing the temperature to remain below 10°C. The material was then centrifuged at 100,000 × *g* for 1 hour, and a fraction that precipitated between 20 and 60% ammonium sulfate concentration was removed. It was then resuspended in a small volume of buffer and dialyzed. This represented the final "20–60" enzyme protein assayed for 5′ nucleotidase activity.

An aqueous extract was made by first mincing and then grinding the fresh tissue with the fine quartz sand in a mortar with equal parts (v/v) of water, kept at 0.4°C. The tissue suspensions were then centrifuged at 14,000 × *g* for 20 minutes. The supernatant fraction was used for assaying of the adenosine deaminase activity.

Enzyme Assays

The 5′ nucleotidase reaction system contained 5 μmoles of the nucleotide (or other phosphorus compound), 50 μmoles of Tris buffer (*p*H 7.5), 10 μmoles of magnesium acetate, and 100 μg of protein in a total volume of 0.5 ml; incubation was for 1 hour at 37°C. The reaction was stopped by the addition of 5% trichloroacetic acid; the mixture was allowed to stand for 1 hour at 0°C, centrifuged at 10,000 × *g* for 10 minutes, and finally decanted. The supernatants were then analyzed for inorganic phosphorus by the Fiske-SubbaRow (*63*) method. Microsomes were made by differential centrifugations between 10,000 and 100,000 × *g* for 1 hour. Liver preparations were treated with the same methods as those of the prostate fractions. All analyses were corrected for phosphorus in the enzyme and substrate materials after heating for 1 hour at 37°C.

The adenosine deaminase reaction mixture contained 2 ml of enzyme fraction (aqueous extract), 2 ml of 0.2 M phosphate buffer, *p*H 7.0, and 10 mg of adenosine. One ml samples were removed after 10, 30, and 50 minutes, stopped with 0.1 ml of 5% TCA, and centrifuged, and subsequently 0.01 ml of each was spotted for chromatographic analysis using Pabst system No. II (*49*). The paper chromatograph spots were eluted with water and the samples were subjected to spectral studies with the Cary 11 spectrophotometer at varying *p*H's, as indicated in the figures. The methods for studying high-speed supernatant preparations from worm and mouse tissues are presented in tables 7 and 8.

TABLE 8.—Nucleotidase activity of C57L/J mouse liver and whole-worm soluble cell fractions (μmoles inorganic phosphorus/1.8 mg protein equivalents of 100,000 × *g* supernatant)*

Nucleotide	Worm	5′ activity	Liver	5′ activity
		2′,3′ activity		2′,3′ activity
5′-AMP	1.107	—	0.086	—
2′,3′-AMP	0.071	1.5	.185	0.46
5′-UMP	.086	—	.140	—
2′,3′-UMP	.035	2.5	.254	0.55
5′-GMP	.177	—	.042	—
2′,3′-GMP	.050	3.6	.096	0.44
5′-CMP	.102	—	.050	—
2′,3′-CMP	.095	1.1	.020	2.5

*Reaction mixture contained 0.1 ml of 100,000 × *g* supernatant of worm (18.5 mg protein/ml) or C57L/J mouse liver (18.36 mg/ml), 50 μmoles Tris, *p*H 7.6, 10 μmoles Mg acetate, 10 μmoles nucleotide as indicated, water to a total volume of 0.5 ml; incubation was for 1 hr at 37° C. The reaction was stopped with 1 ml of 5% trichloroacetic acid; the mixture was allowed to stand for 20 minutes at 0°C, centrifuged at 14,000 × *g* for 10 minutes, and assayed for inorganic phosphorus by the Fiske-SubbaRow (*63*) method. Appropriate blanks containing only supernatant or nucleotide were made.

Results and Discussion

Crude 10,000 × *g* supernatants and precipitates showed activities of approximately 1–1.5 μmoles of inorganic phosphorus liberated per 100 mg wet weight of tissue per hour, using ATP, uridine triphosphate (UTP), CTP, and guanosine triphosphate (GTP) as substrates.

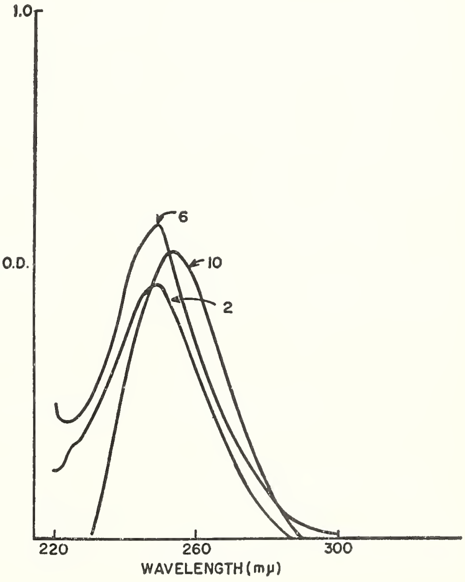
The "20–60" enzyme activity is shown in table 9. Further studies showed little activity using the prostate "20–60" enzyme on 2 : 3 cyclic UMP, poly U, ribose-5′-phosphate, reduced nicotinamide adenine dinucleotide (NADH), glucose-6′-phosphate, or para-nitrophenyl phosphate. Prostate microsomes showed activity for both 2′, 3′, and 5′ mononucleotides. They also attacked 5′ nucleoside diphosphate and inosine triphosphate (ITP), but very little activity was found with 2 : 3 cyclic UMP, poly U, ribose-5′-phosphate, NADH, and glucose-6-phosphate. If, however, the "20–60" enzyme preparation was obtained by vibrating the microsomes, centrifuging at 100,000 × *g*, and making a "20–60" ammonium sulfate fractionation, then virtually all the 3′ nucleotidase activity could be removed. The rat liver "20–60" enzyme showed a similar profile of nucleotidase activity

TABLE 9.—Nucleotide activity of rat prostate (20–60%) ammonium sulfate protein

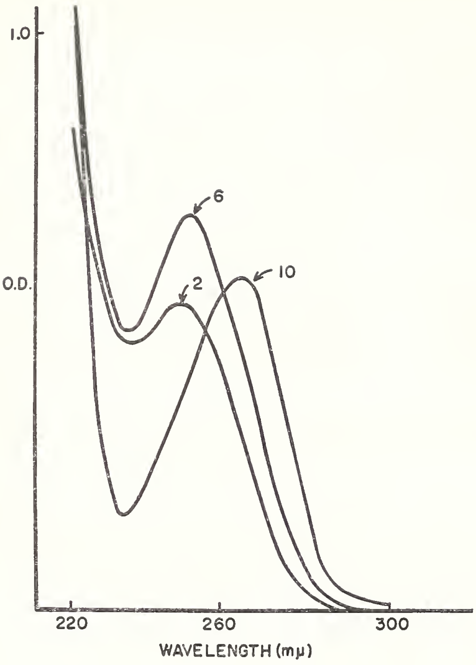
μmoles inorganic phosphorus released per 100 μg protein		μmoles inorganic phosphorus released per 100 μg protein	
Substrate	Per hr	Substrate	Per hr
2′,3′-AMP	0.13	5′-ADP	0.29
2′,3′-CMP	.19	5′-CDP	.51
2′,3′-GMP	.12	5′-GDP	.87
2′,3′-UMP	.02	5′-IDP	1.03
		5′-UDP	.96
5′-AMP	1.20		
5′-CMP	1.54	5′-ATP	.42
5′-GMP	.74	5′-CTP	.46
5′-IMP	.59	5′-GTP	.72
5′-UMP	1.32	5′-ITP	.51
		5′-UTP	.71

except that no activity could be demonstrated for ATP and GTP although UTP and CTP were degraded. This may be due to a large amount of contaminating purine nucleotide phosphokinase activity; however, ITP was effectively degraded.

Aqueous extracts readily deaminated adenosine to a compound with an R_f of 0.53 (theoretical for inosine 0.47) and which gave a 250:260 ratio of 1.68 (theoretical for inosine 1.60) and a 280:260 of 0.25 (theoretical for inosine 0.28) (*see* text-fig. 11). A new spot appeared with longer inoculation times which gave the *p*H shift and absorption characteristics of hypoxanthine (text-fig. 12). It is not known if this latter reaction is due to a purine nucleoside phosphorylase, such as that described by Kalckar (*64*), or a specific inosinase (*65*). Even after prolonged incubations no indication of xanthine oxidase activity could be found. Cytidine was not deaminated to uridine (*66*) by these preparations.



TEXT-FIGURE 11.—Major product of 10-minute enzyme reaction on adenosine; absorption spectra on material eluted from portion of chromatogram having an R_f matching that of an inosine control. Numbers indicate *p*H.



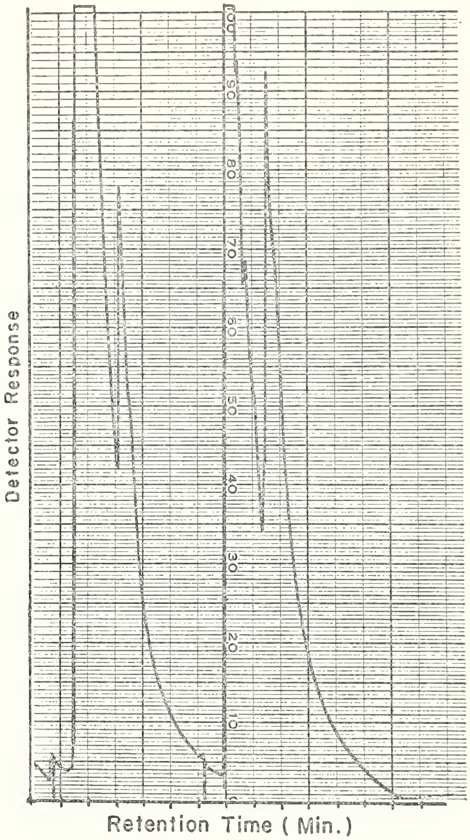
TEXT-FIGURE 12.—Major product of 50-minute enzyme reaction on adenosine; absorption spectra on material eluted from portion of chromatogram having an R_f matching that of a hypoxanthine control. Numbers indicate *p*H.

Worm high-speed supernatent cell fraction was studied for its specificity of nucleotidase activity and is compared with mouse liver nonparticulate cell fractions in table 8. Worm cell activities were relatively large on the 5′ nucleotides as contrasted to the 2′, 3′ nucleotides, whereas mouse liver was more active with 2′, 3′ substrate material than 5′ except for CMP.

Earthworm adenosine triphosphatase studies have been reported elsewhere (*67*). The writer has been studying nucleosides with gas chromatography (*68–70*). It is somewhat surprising to find that nucleoside phosphates will pass through columns after being silylated. Silylation substitutes a trimethylsilyl function for potentially ionizable hydrogen and results in fewer polar molecules. Hashizume and Sasaki (*71*) discovered that phosphoric acid itself could be analyzed by gas chromatography, and this was applied to extracts of worm tissue. Text-figure 13 shows that phosphate (along with several other more minor peaks of unknown substances) can be detected in these preparations by this method and is probably its first application to a biological preparation.

COMMENTS ON THEORETICAL ASPECTS

The finding of giant nuclei in the pharynx of earthworms and the subsequent ability to induce these with carcinogens, coupled with the finding of giant nuclei in a pharyngeal neoplasm of worms, led to further examinations of the nucleolar organizer regions of the interphase DNA



TEXT-FIGURE 13.—The analysis of inorganic phosphate by gas-liquid chromatography, trimethylsilylated sodium phosphate (*left*), and trimethylsilylated extracts of *Lumbricus* tissue (*right*).

which appears to be the site for rRNA synthesis and possibly tRNA. Many speculations could be offered for a correlation with carcinogenesis, such as planar hydrocarbons intercalating with specific regions of the chromatin DNA which thereby has an altered surface and inhibits histone attachment and repression of a templating area. This alters the control of synthesis of species of RNAs not normally produced or produced in greater abundance than usual. However, the possibility that sRNA alkylation is basic in carcinogenesis, which results in the appearance of a genetic lesion, has been proposed by Axel *et al.* (*72*). Work in my laboratory has been devoted to studies on the sRNA methylase system of Borek in neoplastic tissues. No invertebrate neoplasm has as yet been investigated. In general, neoplastic tissues have more sRNA methylase activity than normal adult tissues. Tsutsui *et al.* (*73*) and Hancock (*24, 26*) have found that the extent of methylation of a given amount of sRNA is greater when cell fractions from neoplastic cells are used. Thus there must be new sites that are methylated, and new enzymes, or at least alterations in enzyme activity to those normally present. Furthermore, Tsutsui *et al.* (*73*) have demonstrated new bases becoming methylated with the use of enzymes from neoplastic sources. It would be interesting to find that invertebrate tumor preparations were capable of extensively hypermethylating. This would be analogous to the situation described for mammalian tumors.

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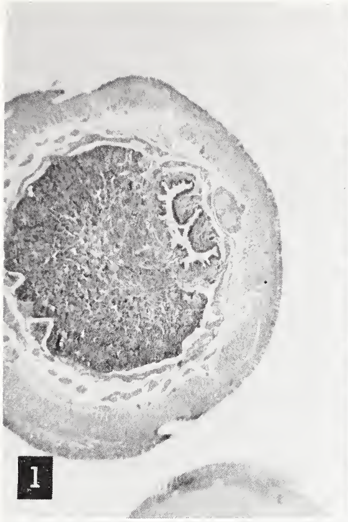
Figures 1 and 2.—Pharyngeal gland; figure 3.—pharyngeal epithelium; figures 4–9.—pharyngeal epithelium with giant nuclei after 40 days of MCA treatment.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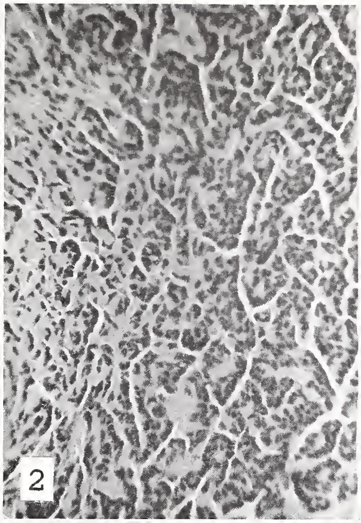
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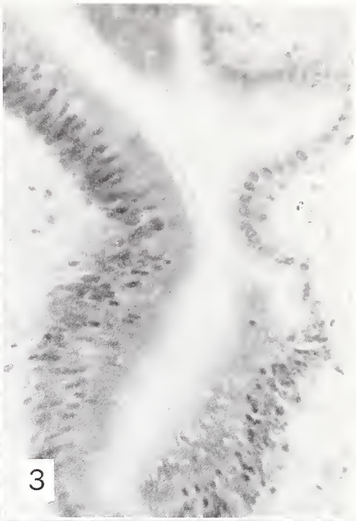
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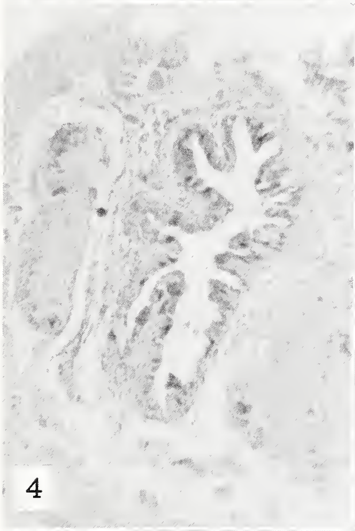
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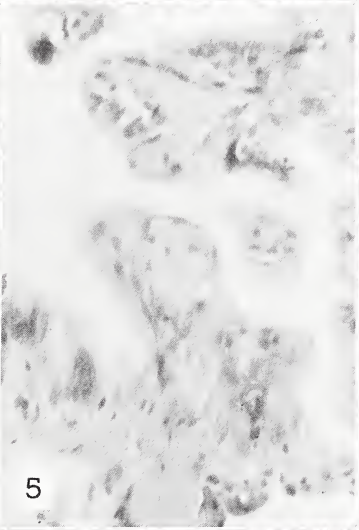
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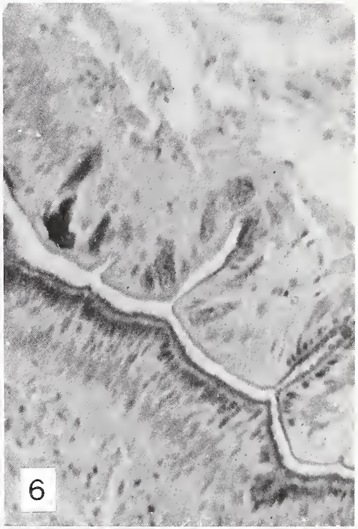
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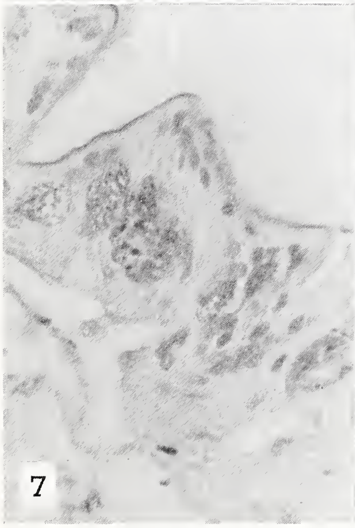
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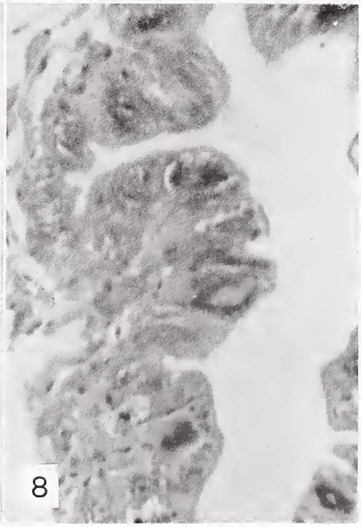
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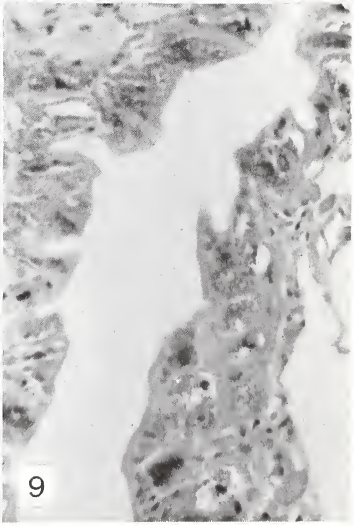
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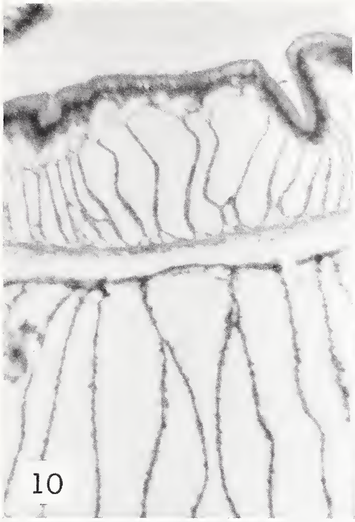
Figures 10 and 11.—Calciferous gland; figure 12.—gizzard; figure 13.—seminal receptacle; figure 14.—heart; figure 15.—nephrostome; figure 16.—typhlosole-oil red O stain; figure 17.—dermis (*upper left*), and circular muscle, longitudinal muscle, and crop (*lower right*); figure 18.—nephridium.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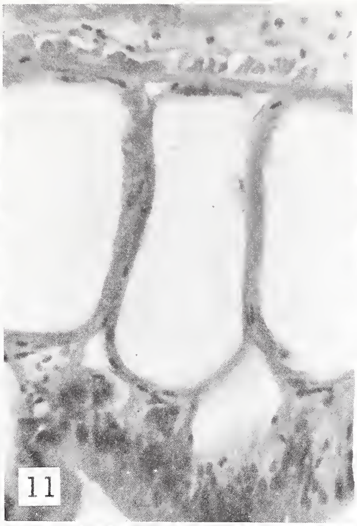
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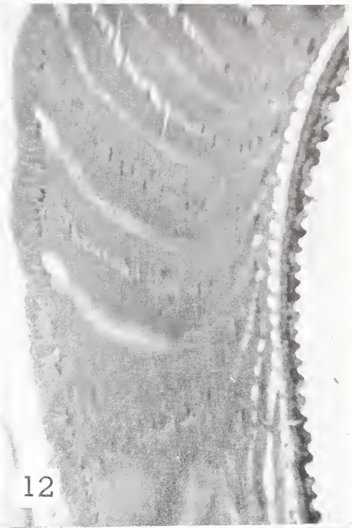
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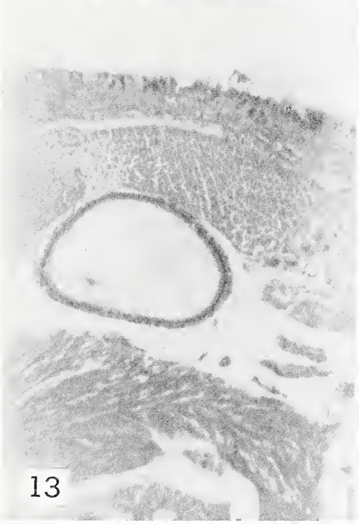
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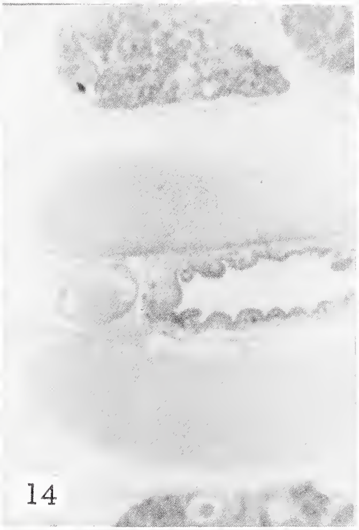
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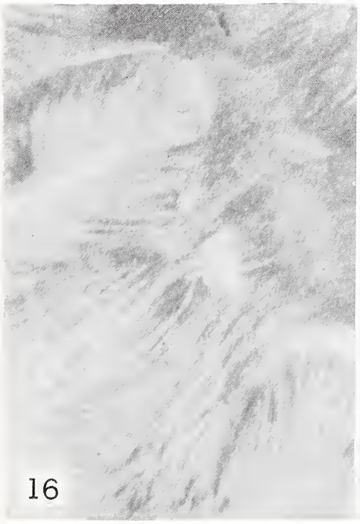
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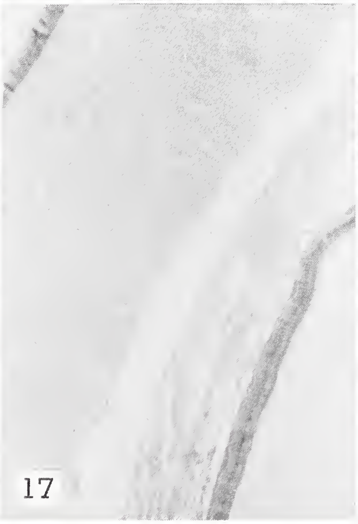
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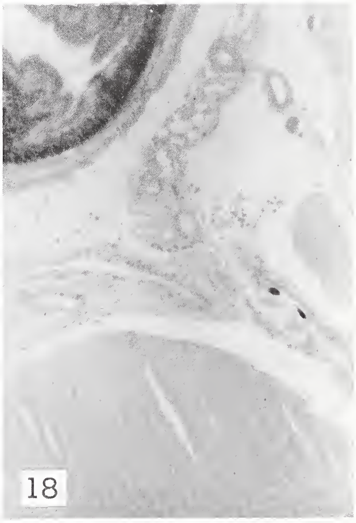
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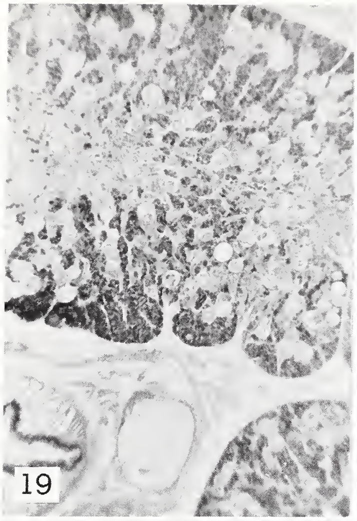
Figures 19 and 20.—Seminal vesicle; figure 21.—seminal vesicle after 29 days of MCA paintings; figure 22.—monocystis; figures 23–25.—cyst in the seminal vesicle of an untreated worm; figure 26.—cyst, periodic acid-Schiff stain; figure 27.—seminal funnel.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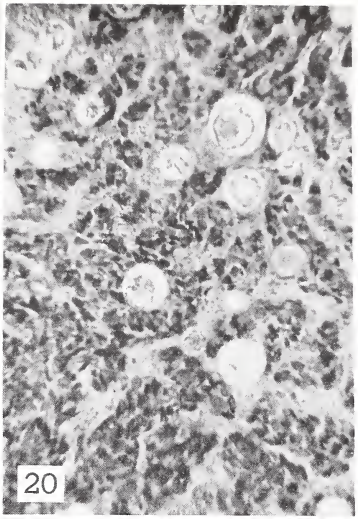
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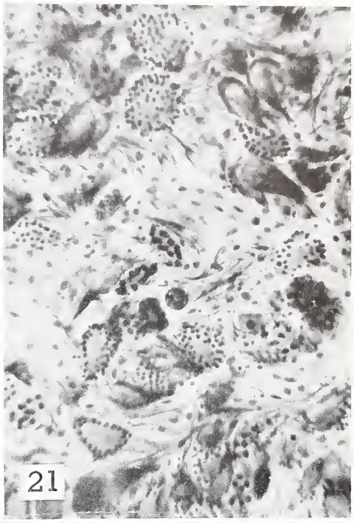
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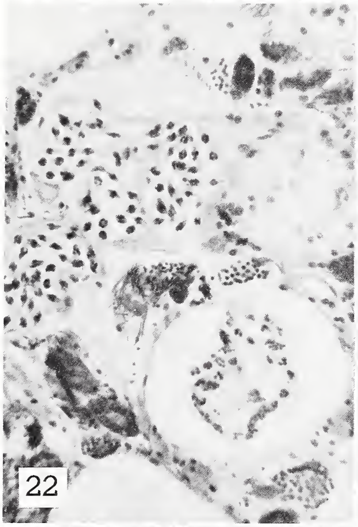
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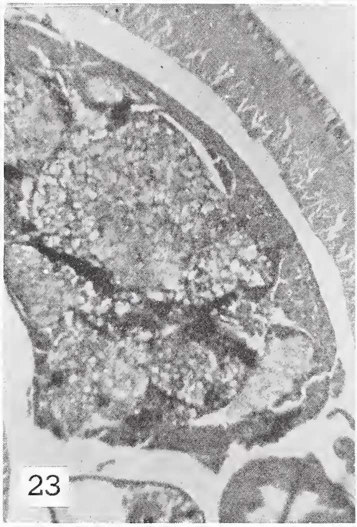
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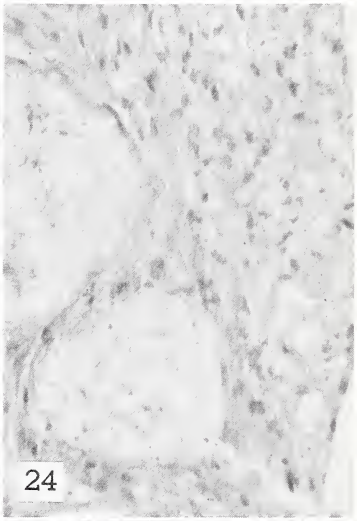
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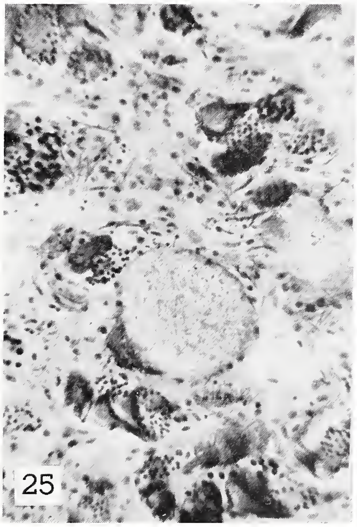
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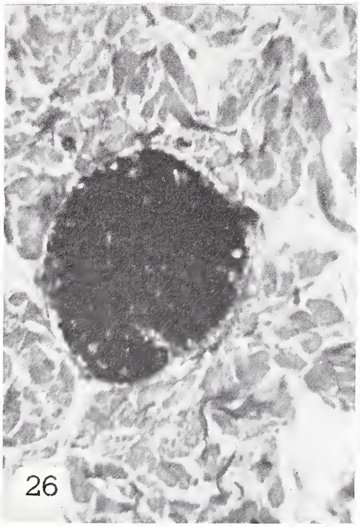
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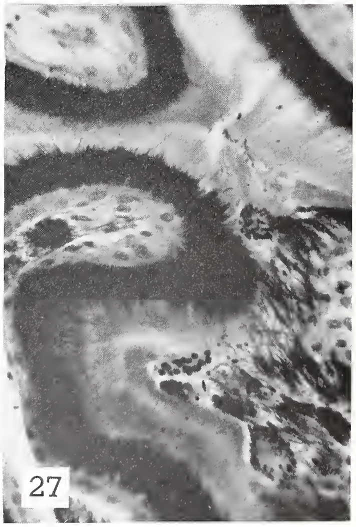
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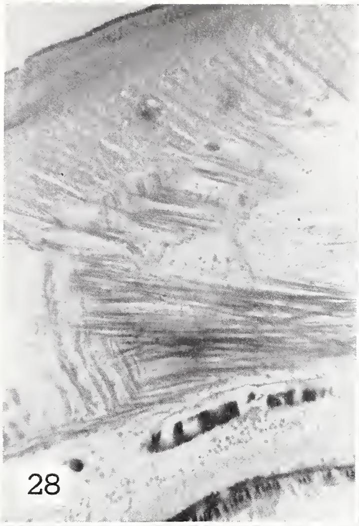
Figure 28.—Septal muscle fibers; figure 29.—septae; figures 30–32.—dermis; figures 33–35.—testis; figure 36.—testis in worm after 40 days of MCA paintings.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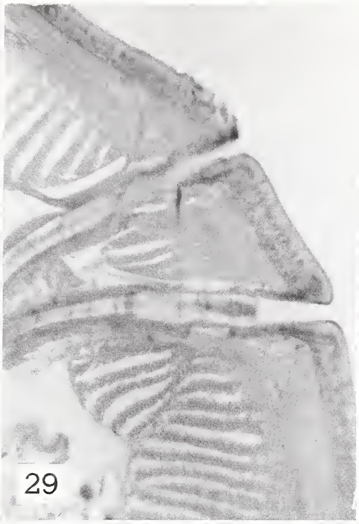
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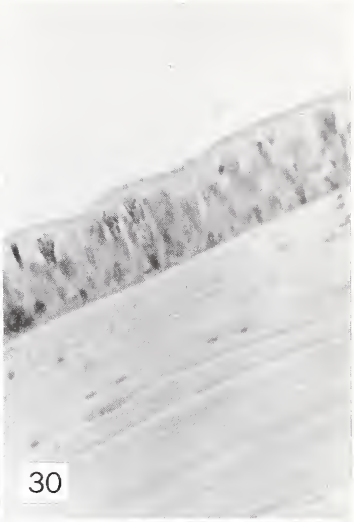
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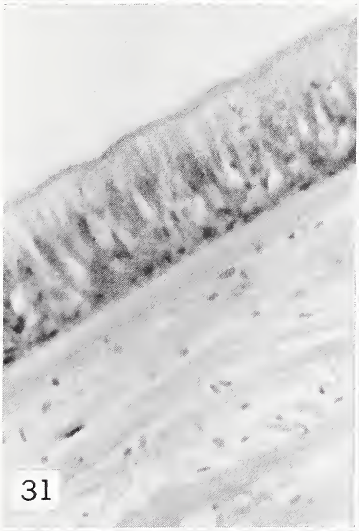
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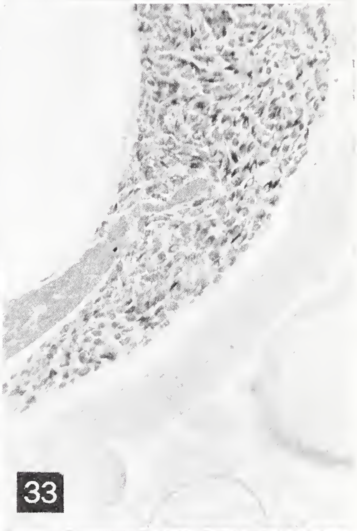
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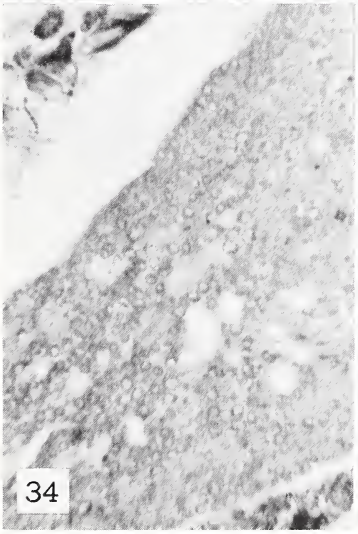
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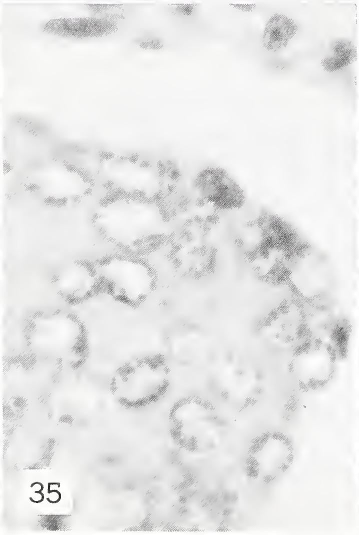
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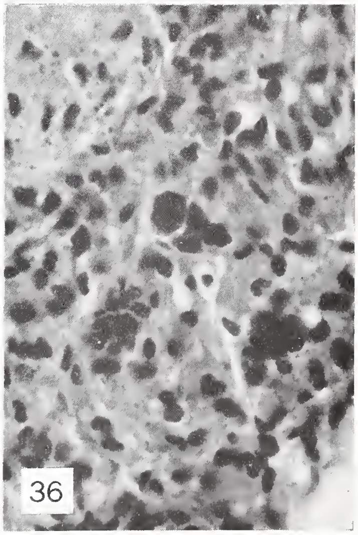
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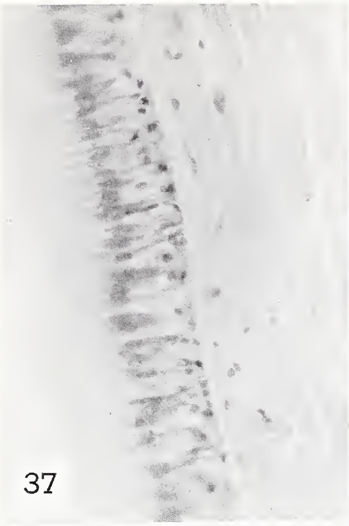
FIGURE 37.—Dermis and circular muscle; figure 38.—dermis and circular muscle, Sudan B. stain; figure 39.—dermis of worm after 22 days of MCA painting; figure 40.—dermis of worm after 25 days of MCA paintings; figure 41.—dermis of worm after 22 days of MCA paintings; figure 42.—clitellum of worm after 45 days of MCA paintings; figure 43.—clitellum; figures 44 and 45.—small, densely staining nucleated cells proliferating beyond original border of worm, after 45 days of MCA painting.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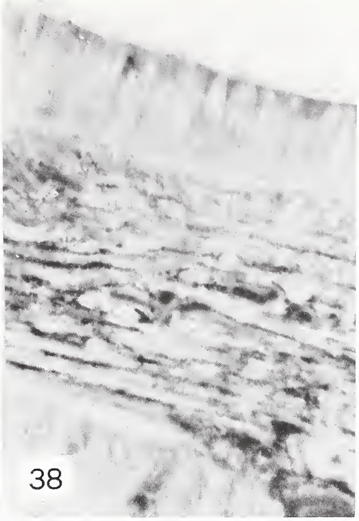
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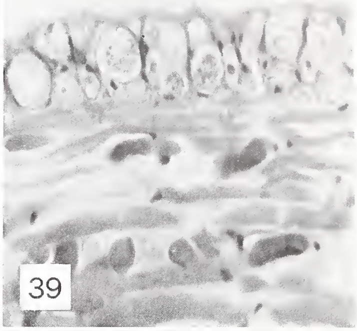
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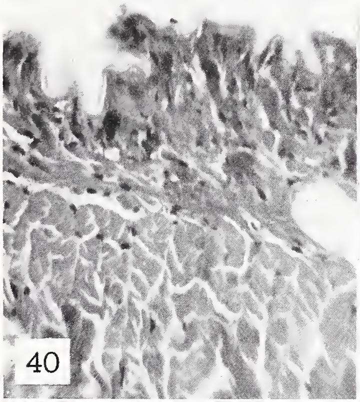
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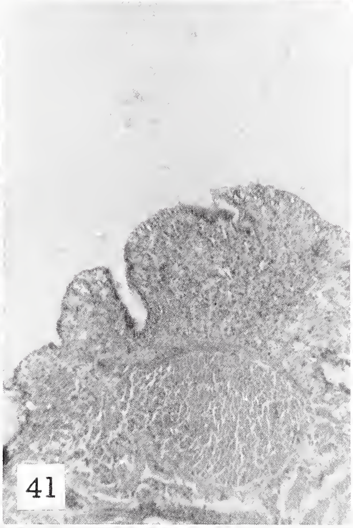
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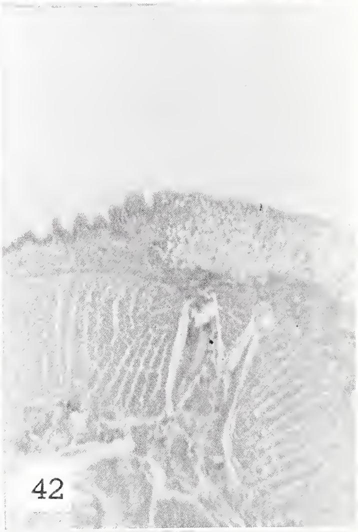
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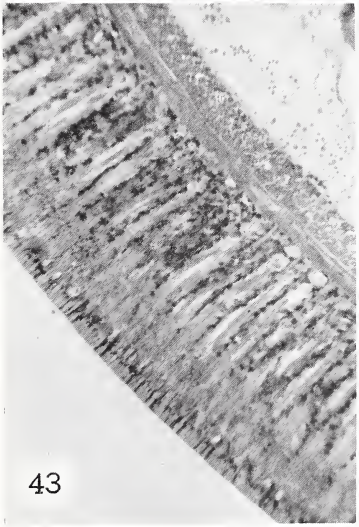
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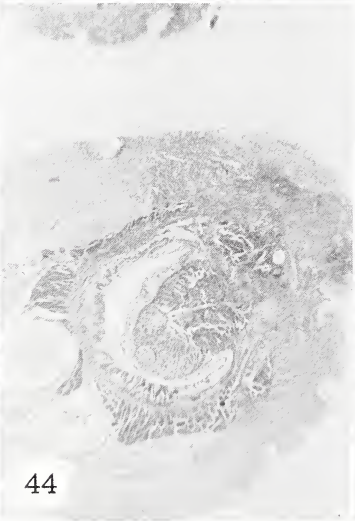
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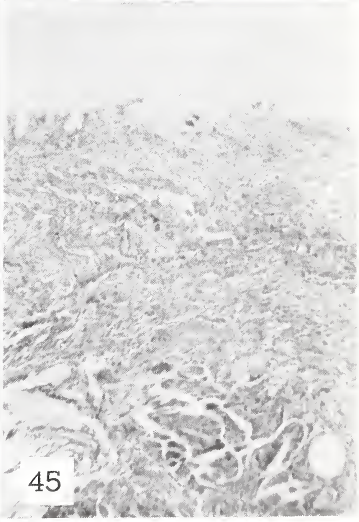
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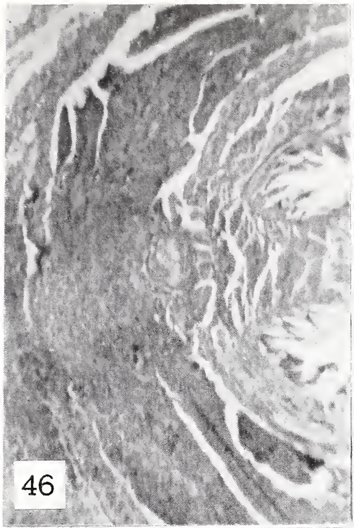
FIGURES 46, 48, and 49.—Chloragogen cell tumor in worm after 15 days of MCA paintings; figure 47.—chloragogen cells; figure 50.—typhlosole; figure 51.—septum; figure 52.—esophageal mass in untreated worm.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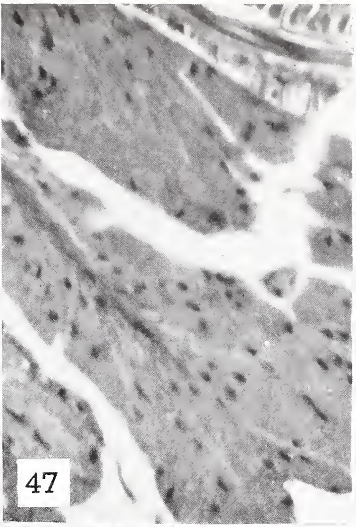
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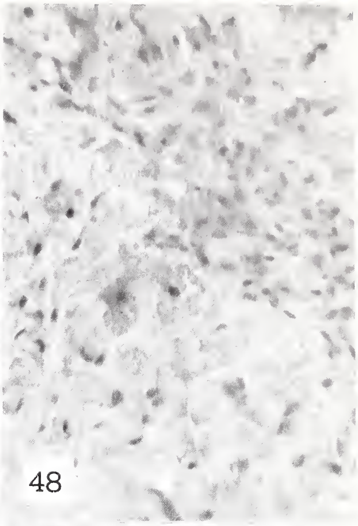
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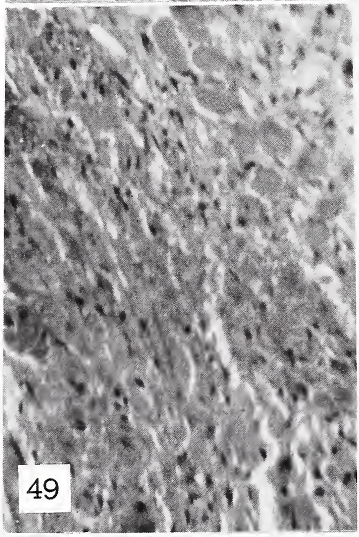
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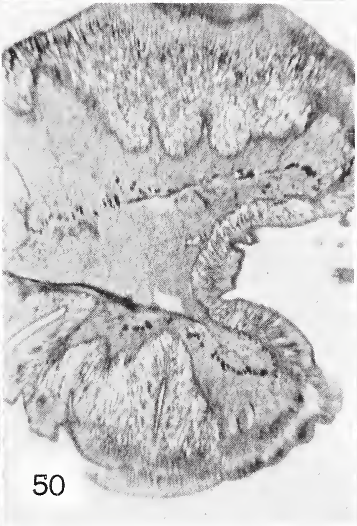
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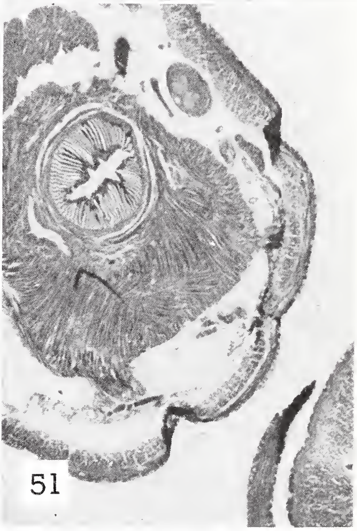
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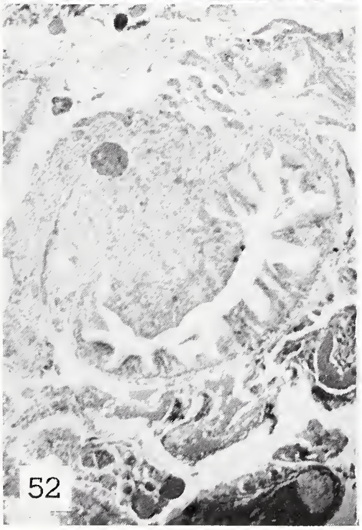
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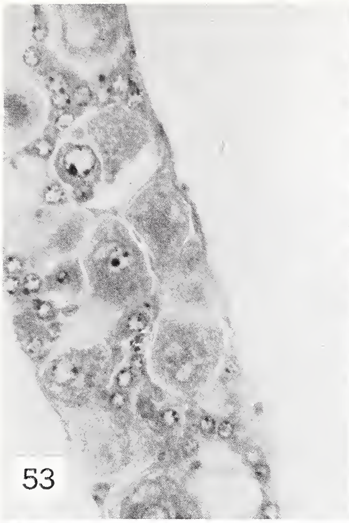
Figure 53.—ovary; figure 54.—ovary in worm after 19 days of MCA paintings; figures 55 and 56.—ovary of worm after 55 days of MCA paintings; figures 57 and 59.—testis of worm after 40 days of MCA paintings; figure 58.—normal testis; figure 60.—testis in worm after 55 days of MCA paintings; figure 61.—testis in worm after 40 days of MCA paintings.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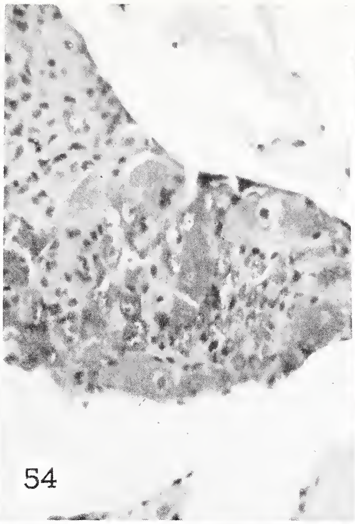
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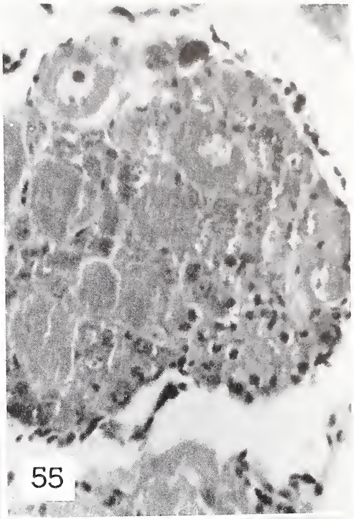
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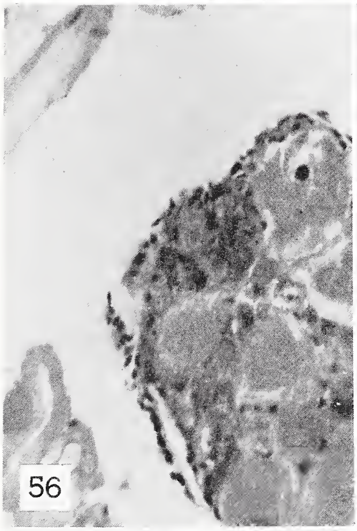
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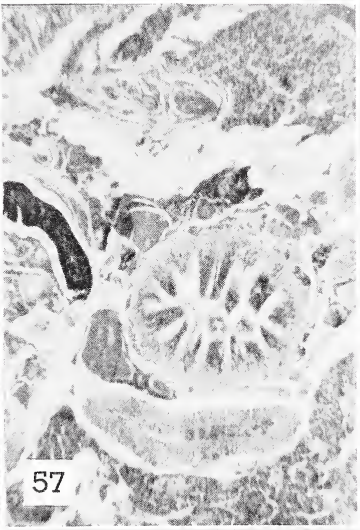
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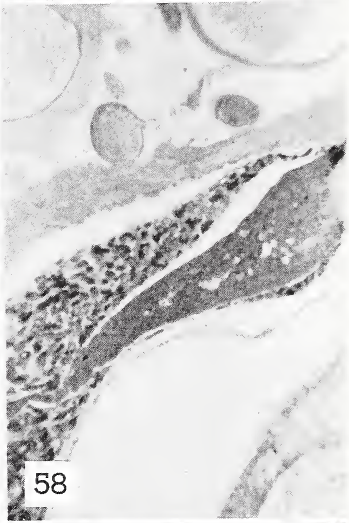
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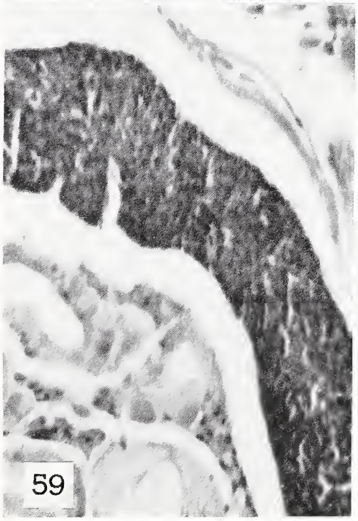
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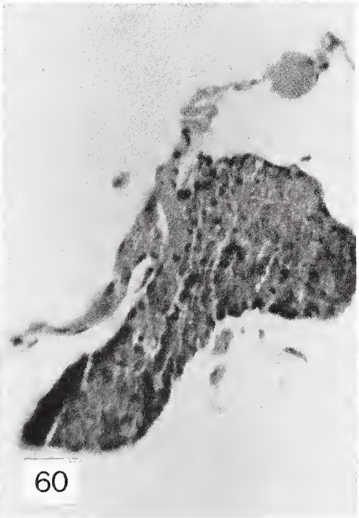
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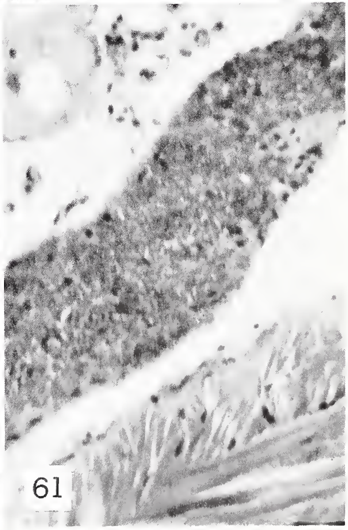
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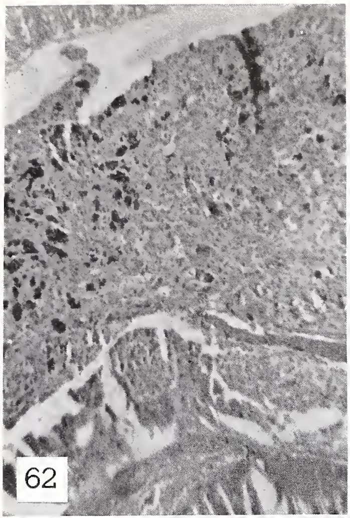
FIGURE 62.—Variant of seminal vesicle cellular content in untreated worm; figure 63.—seminal vesicle in worm after 19 days of MCA treatment; figure 64.—seminal vesicle in worm after 40 days of MCA treatment; figures 65–69.—unusual tumorous mass in seminal vesicle; figure 70.—sperm, methyl green-pyridine stain.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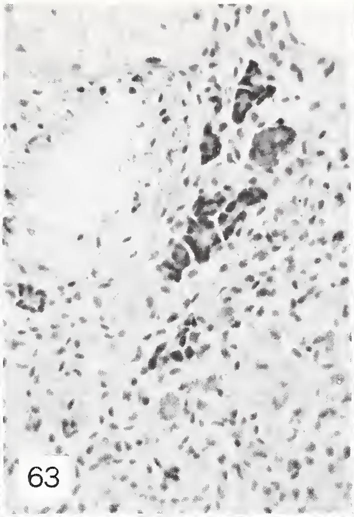
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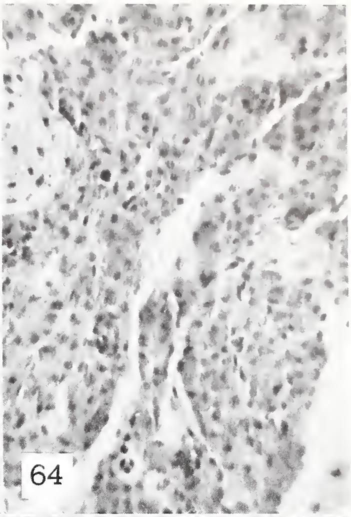
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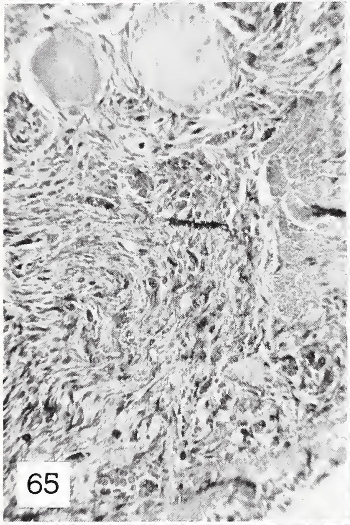
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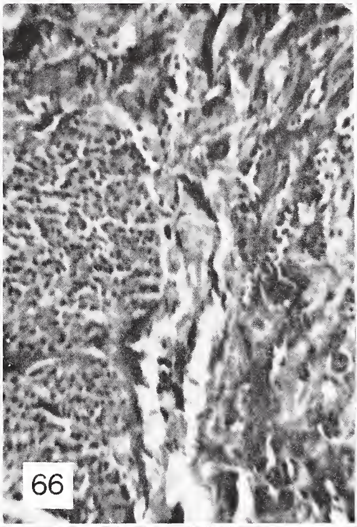
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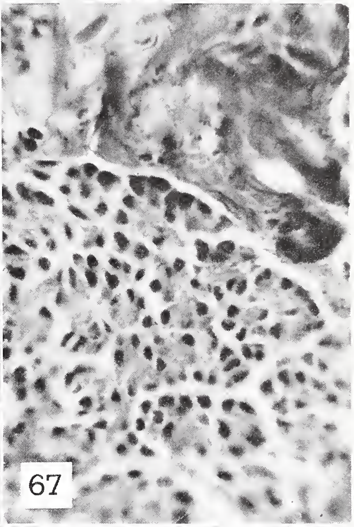
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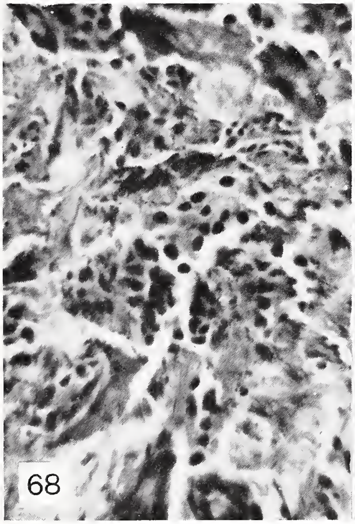
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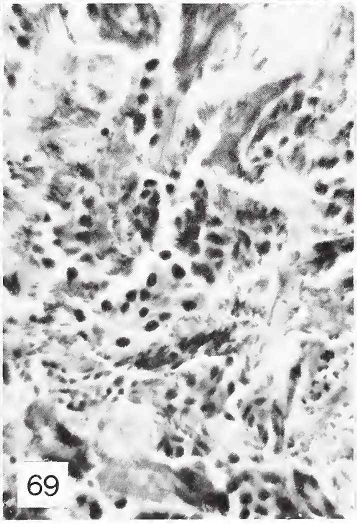
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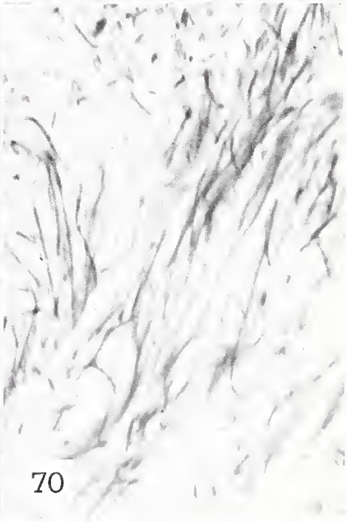
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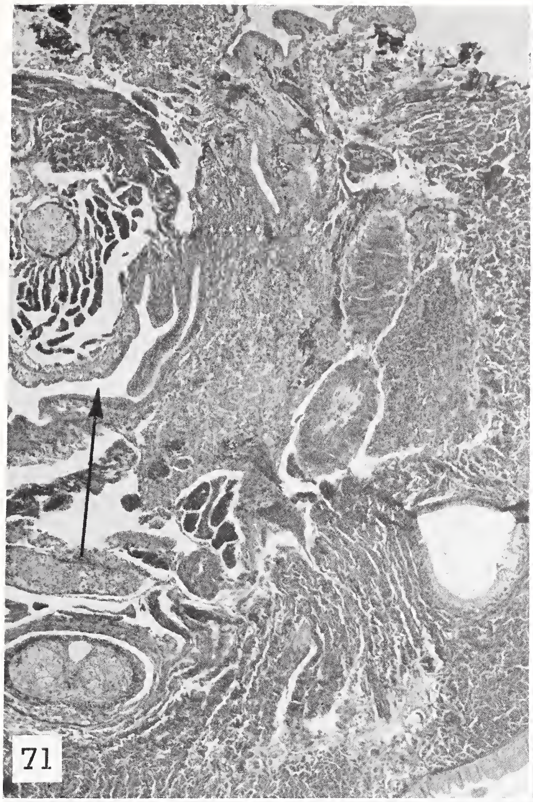
Figure 71.—Cross section of worm at level of pharynx (*arrow*) 23 days after having received 100 r; figure 72.—Coelomic mass (*arrows*) in irradiated worm treated as in figure 71; figure 73.—multinucleated giant cell in coelomic cavity.



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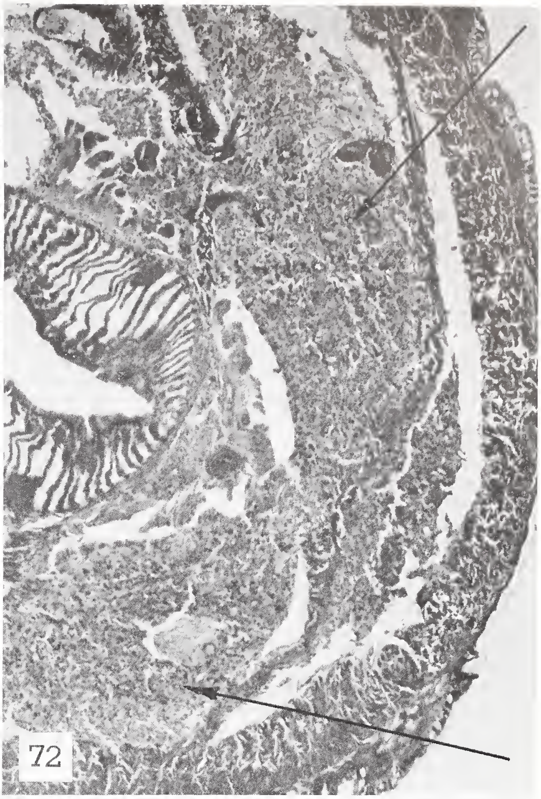

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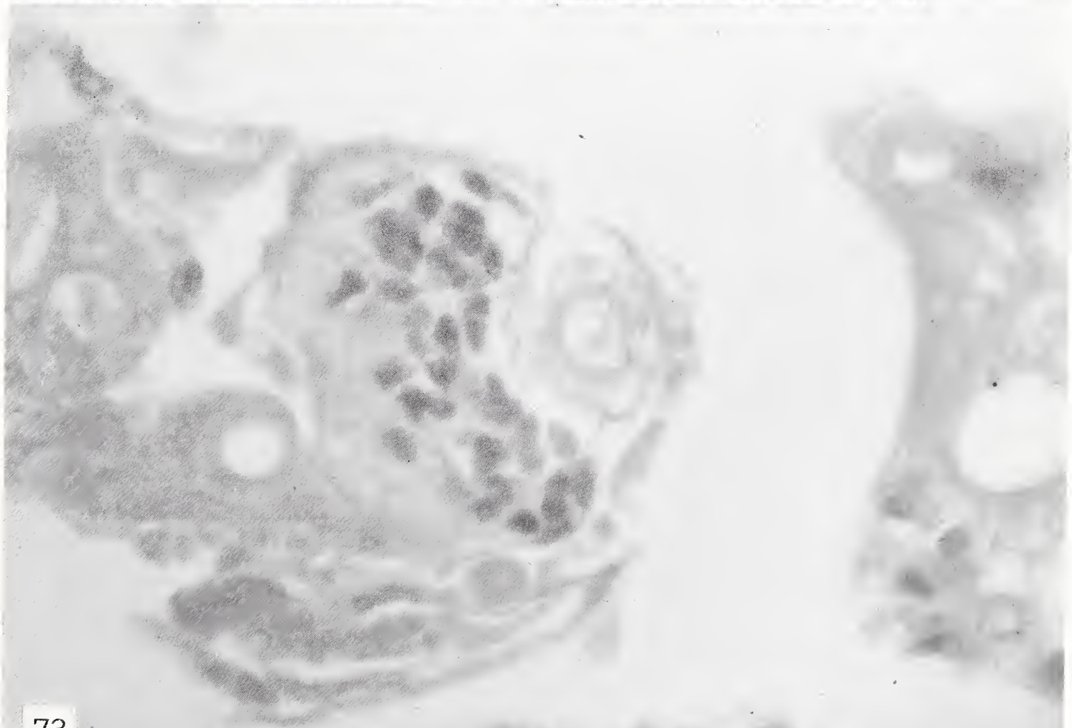

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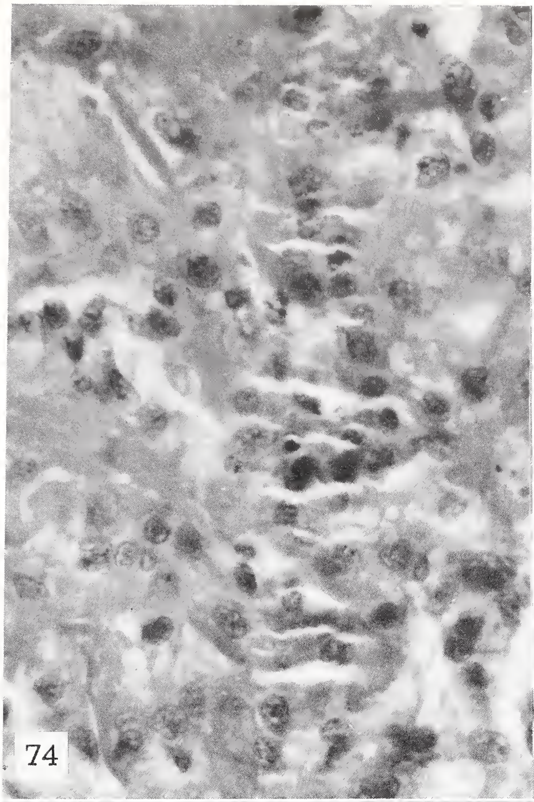
Figures 74 and 75.—Cells of coelomic mass in worms 23 days after receiving 1000 r; figures 76 and 77.—dorsal proliferating mass of worm 12 days after receiving 1000 r.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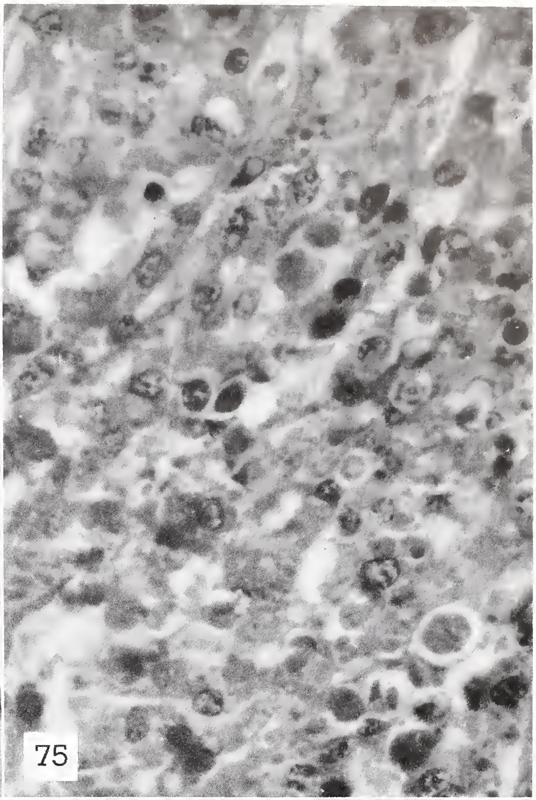
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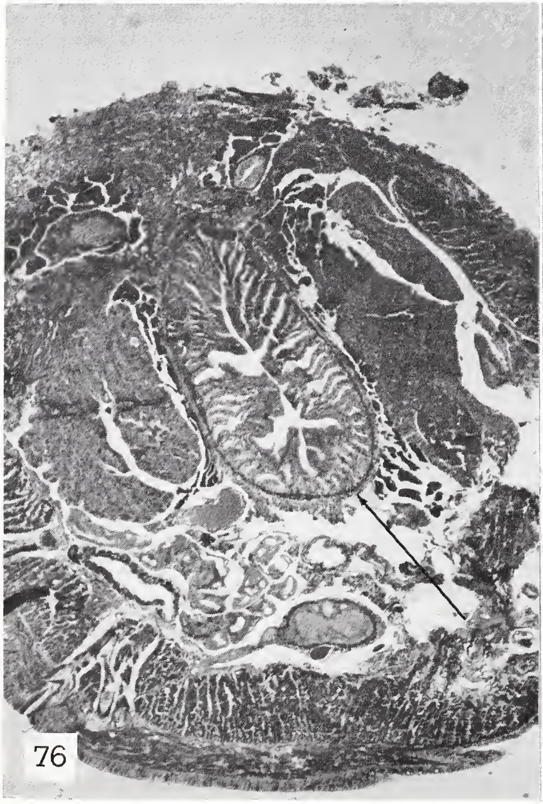
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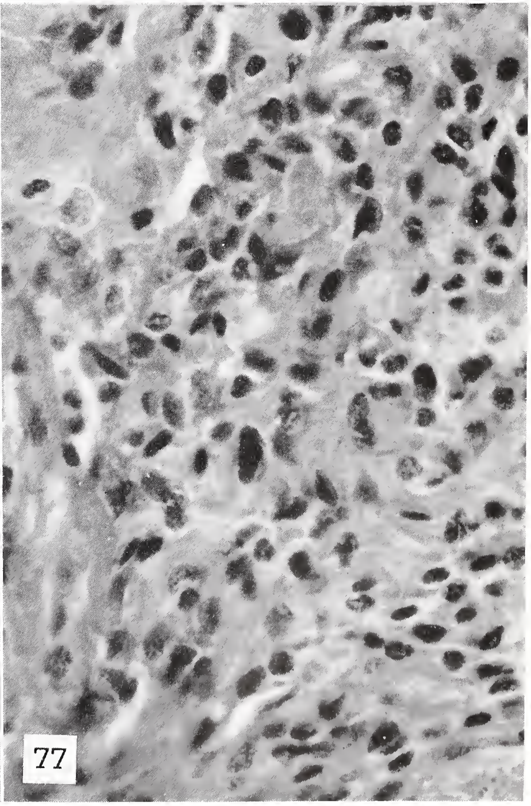
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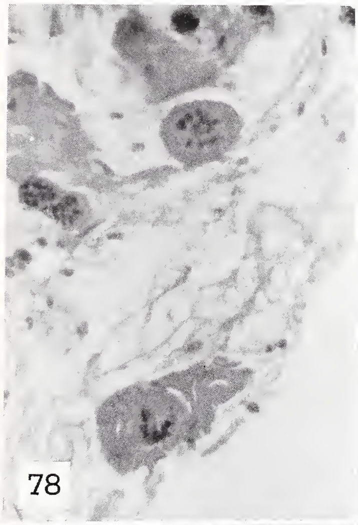
FIGURE 78.—Giant cell in worm after 22 days of MCA paintings; figures 79 and 80.—giant cells 20 days after the induction of a burn lesion; figure 81.—amebocytes; figure 82.—amebocytes collected around a thread saturated with MCA.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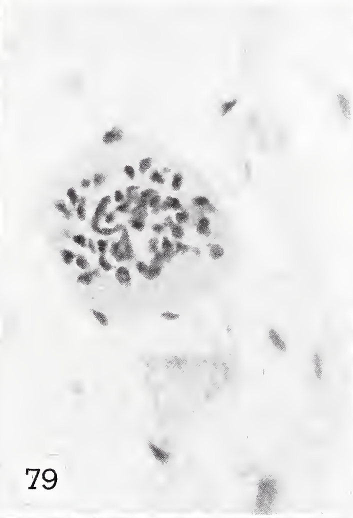
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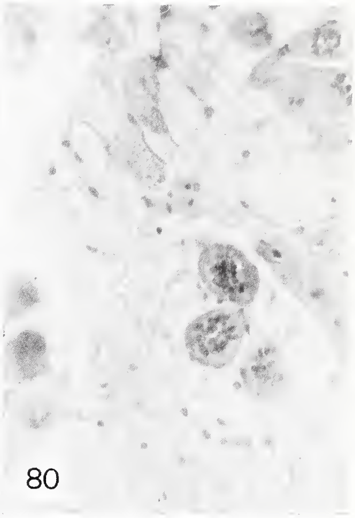
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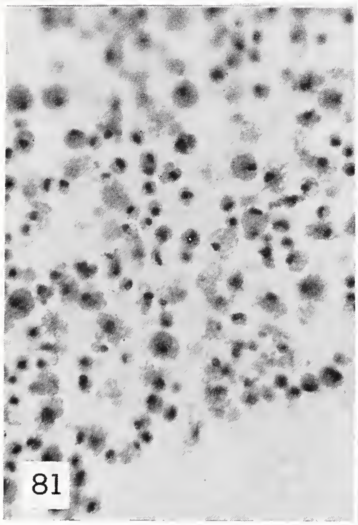
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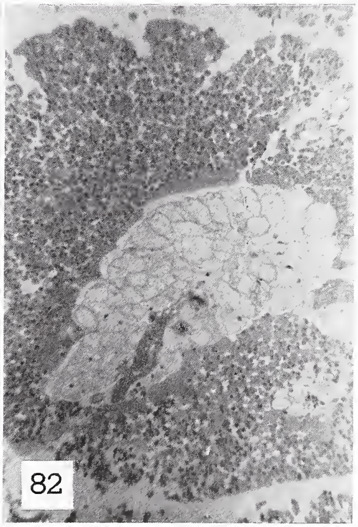
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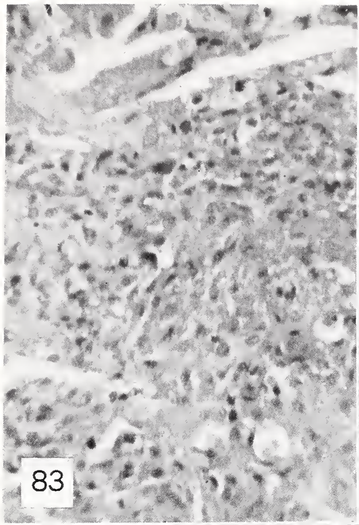
Figures 83 and 84.—Regeneration cells in 20-day-old burn area; figure 85.—mycotic mass, periodic acid-Schiff stain; figures 86–88.—"ascites-like neoplastic" cells in worm after 22 days of MCA paintings; figure 89.—cells of undetermined origin in an anomalous mass in a worm after 30 days of MCA paintings; figure 90.—an anomalous group of muscle cells in an untreated worm; figure 91.—mass on dermis of worm after 56 days of MCA paintings.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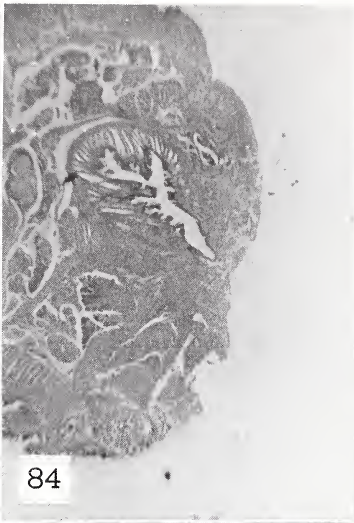
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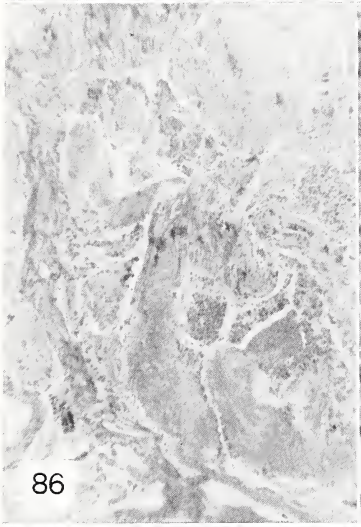
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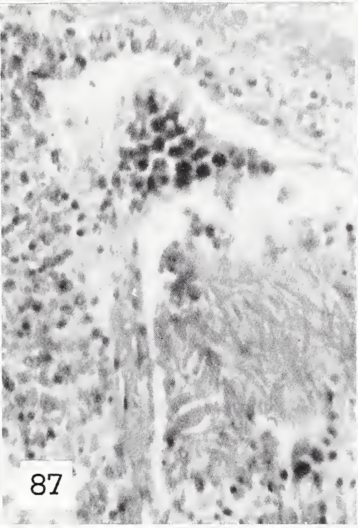
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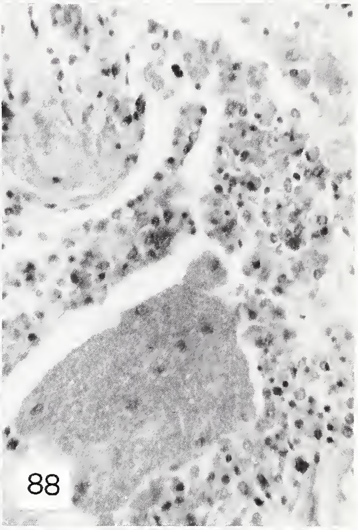
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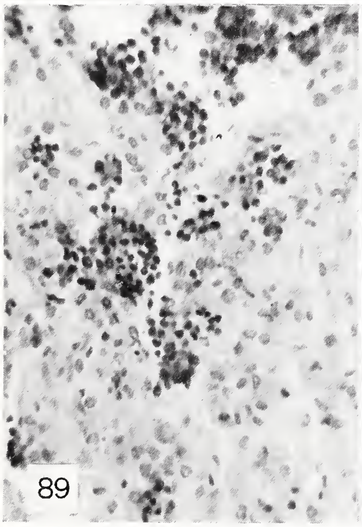
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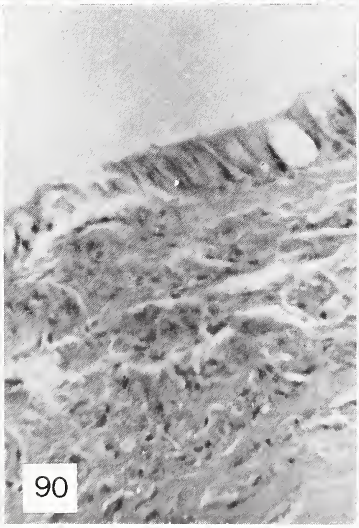
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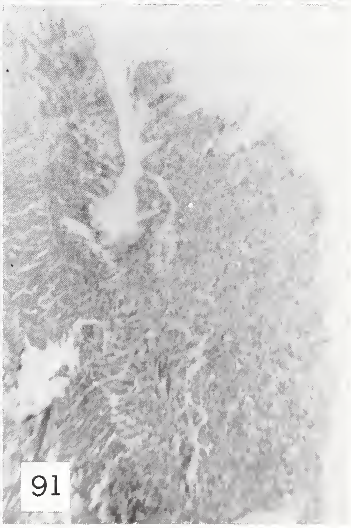
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Epithelioma-Like Lesion in *Lumbricus terrestris* After Cold Injury

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SUMMARY

An area approximately 4 mm in diameter on the dorsum of *Lumbricus terrestris* was exposed to contact with CO_2 snow for 20 seconds. This produced a "standard" wound characterized by necrosis and sloughing of the superficial epithelium, and a break in the basement membrane followed by rapid proliferation of adjacent surface epithelium which poured in to fill the defect, producing a small "button" of epithelial tissue. The new cells, though fairly regular in morphologic characteristics, appeared to invade between the circular muscle and into the deeper longitudinal muscle; some even reached the internal viscera. As long as 3 months later, the surface epithelium had healed, but there was still a break in the basement membrane and the epithelial cells persisted in the deeper layers. Early there was some attempted regeneration of muscle, but this was completed in only a few days. There was some increase in collagen about the muscle bundles, apparently elaborated by the muscle cells. No fibroblasts were recognized, and instead of a scar there was a filling-in with surface epithelial cells, which persisted but did not continue to spread and appeared "controlled" in their behavior.—Nat Cancer Inst Monogr 31: 645–653, 1969.

A MARKED difference in the hydroxyproline and proline of collagen from the cuticle and from the body of earthworms was recently reported (*1*). In the same study a similar intraspecies difference was indicated in *Ascaris*, and subsequent reports have confirmed the occurrence of less-marked intraspecies variation in the composition of vertebrate collagens (*2*, *3*). It seemed of interest to learn whether a healing wound in the body wall of earthworms would be repaired with collagen of composition similar to that of the cuticle or of the subcuticular tissues. Surprisingly, a significant collagen deposition did not occur after tissue damage by freezing; fibroblasts failed to appear and instead the wounds were closed by a proliferation of epithelium which, by the standards of mammalian pathology, had some of the characteristics of a neoplasm. This report describes the histologic features of the epithelioma-like repair of the tissue damage.

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Mature *Lumbricus terrestris*, approximately 10 cm long, were purchased from Carolina Biological Supply Company, Burlington, North Carolina. At the time of use the worms were washed with running tap water and placed in a beaker. Each worm was spread linearly on parafilm (R) and a separate piece of parafilm was placed over the dorsal surface. A 5 ml beaker, partly filled with finely crushed solid CO_2, was applied firmly to the parafilm over the dorsal surface of the worm about one-third the length of the worm from the tip of the tail. The cold beaker, thus applied to the parafilm-covered worm, was maintained for 20 seconds as indicated by a stopwatch. Initially, the surface of the worm turned an opaque yellowish-white where contacted (fig. 1), but then quickly faded as the area thawed so that the site of injury was no longer evident. Excessive mucus was secreted immediately after the injury. The worms were stored in the cold room at 4°C in the dirt-laden moss bedding in which they were supplied.

All worms were examined daily. Two worms were killed at daily intervals for the first 2 weeks and then less frequently. After a few days, the injured area appeared dull, drier, and paler tan than the adjacent normal area. The segments involved seemed slightly shortened, and there was a slight bilateral protrusion, which apparently did not change caliber with the adjacent segments as the worm moved. The dorsal surface seemed neither to protrude nor recede where injured. In some worms, the area of injury was slightly indurated and could be palpated as a small, firm plaque or button. After 8–10 days, the site of injury consisted of a light-tan "button" of tissue visible beneath the smooth, glistening surface and bulging laterally on each side. The segments involved were slightly shortened and did not stretch like the normal segments with the progressive undulating movements that characterize the normal locomotion of the earthworm. The dorsal surface seemed entirely healed with, in some animals, a slight umbilication marking the center of the underlying "button," indicating the previously injured area. The ventral surface was quite normal. This appearance was maintained for as long as the worms were followed, up to several months.

Worms were killed by dropping them in Bouin's solution. The previously injured segments of the worms were fixed in Bouin's solution for 18 hours, then a longitudinal perpendicular section was made through the lesion, and the tissue was passed through several changes of 70% alcohol until the yellow picric acid had almost disappeared. The tissue was subsequently embedded in paraffin in the usual manner and sections were cut 5 μ thick. The sections were stained with hematoxylin and eosin, periodic acid-Schiff (PAS), and Heidenhain and by reticulum techniques.

Microscopic examination of the mildest lesions on the first day revealed that the cuticle was lifted off and there was vacuolar swelling at the base of the epithelial cells, with linear distortion of nuclei from below or from the side by the vacuoles. At times the cells were ruptured. With progressive damage the cells became a granular amorphous mass containing randomly scattered small, round nuclei. In places the epithelium tended to be lifted off of the underlying basement membrane. In the milder lesions the basement membrane was intact. Beneath the basement membrane, particularly in the lower layers of less-compact circular muscle, the previously inconspicious cytoplasm of the sarcolemmal cells became evident and tended to form basophilic rings around the eosinophilic sarcoplasm, and multiple nuclei were seen in the plane of the section.

Between the muscle bundles of the deeper longitudinal muscle, groups of cells appeared that seemed to be infiltrating from the coelomic cavity below (fig. 4). These at times migrated along radial septae which inserted at the incisurae and compartmentalized the dorsal coelom. In the underlying coelomic cavity there was an aggregation of loose and clustered amebocytes, the leukocytes of the earthworm, which are often found around the blood vessels in the chlorogogue tissue around the enteric tube (*4*, *5*). In a few instances degenerating eleocytes were also seen (fig. 4), but they were not prominent in most worms.

In the more severe lesions on the first and second days, the basement membrane was ruptured (fig. 3) and the granular degeneration extended into the circular muscle layer; there was an intermingling of the epithelial cells from the surface and the "activated" muscle cells. The regeneration in the overlying and adjacent epithelium appeared to arise from the "basal cells" of the epithelium (*6*) and was somewhat squamous or flattened and less organized than the orderly columnar cells (fig. 2). Despite the apparent proliferative nature of this process, mitotic figures were not seen. Continuing from the 3d–6th day after freezing, the cells poured through the break in the basement membrane, filling the defect in the underlying circular muscle and, at times, deeper longitudinal muscle; the edges of the basement membrane were turned in, and the reticular network, which normally surrounds every muscle fiber, was swept aside or lost. Solid masses of somewhat spindle-shaped epithelial cells filled the area. These cells were arranged rather haphazardly, and often extended unrestrained between the adjacent muscle fibers, resembling infiltration by tumors. About the edge, there were occasional fragments of reticulum or trapped muscle fibers in the otherwise solid mass of epithelial cells.

Ordinarily by the 7th day, the mass of cells replacing the muscular layers had a surface layer of epithelium with a single layer of cells fairly well oriented and covered with cuticle, but the mucus-producing cells were seen only at the edges and had not yet returned in this regenerated area. A few trapped muscle fibers were seen near the surface in the epithelial mass. Figures 5 and 6 illustrate a 14-day lesion. In a particularly deep wound at 14 days, the epithelial growth extended even into the underlying viscera and involved the wall of the enteric tube, and the surface layer had not yet returned to columnar form and cuticle did not completely cover the area. In this worm there was an aggregation of degenerating and necrotic eleocytes adjacent to the involved gut.

In a specimen examined 22 days after freezing, the epithelial growth had extended into the body cavity, appearing to spread within the cavity. As always in isolated specimens, it was difficult to tell whether the cells were infiltrating from the epithelium inward or from the body cavity outward, or both. However, the sequential sampling indicated clearly an inward growth of epithelium with an early leukocytic response which soon died out. At 22 days there was no remaining leukocytic response of the host, and leukocytes were scarce around the blood vessels in the chlorogogue tissue or in the coelomic cavity. After 1 month there was still a thickening of the epithelium in the formerly injured area, and the surface carried normal cuticle (fig. 7). Examination of the surface cells revealed that many of the cells contained PAS-positive, diastase-resistant particles, but the typical goblet cells had not returned.

These characteristics of the lesion, though tending to diminish somewhat with time, were maintained both grossly and microscopically as long as the worms were followed, *i.e.*, up to several months.

DISCUSSION AND CONCLUSION

Our findings, that epithelial proliferation constitutes the mechanism of wound healing in the earthworm, are consistent with the absence of fibroblasts, as suggested by a recent electron microscopic study (*6*). The collagen of the species is probably derived from epithelial and muscle cells. This generation of collagen from other than fibroblasts may correlate with the chemical findings of high hydroxyproline/proline ratios. The epithelial regeneration induced by freezing injury effectively replaces the damaged tissue and, as in mammals, the skeletal muscle shows an abortive regeneration.

The early leukocytic response arising from the coelomic cavity apparently serves to provide phagocytes to remove damaged cells and debris, but it seems quite evanescent and rather soon recedes. It does not remain to form tumor-like masses, as has been suggested by Hancock (*7*). Indeed, the tumors described by Hancock may be merely healing wounds. Hancock's "granular cell myoblastoma" may well represent merely the epithelial proliferation resulting from a burn which he inflicted before painting with 3-methylcholanthrene. No controls were mentioned and therefore it is questionable as to what the 3-methylcholanthrene may have contributed to the lesion. Subsequent longer-term painting experiments, without a preliminary injury, by Cooper (*8*) elicited merely an epithelial thickening and a mild inflammatory response.

The epithelial regeneration has certain characteristics of a neoplasm in mammals, particularly by the formation of a rather disorganized "nonfunctioning" mass of cells which infiltrates the adjacent normal tissues and persists as a mass long after the injury. Other features are missing such as variability of size, shape, and staining of cells, continuing progresssive growth with displacement or destruction, and ulceration of the surface. Thus, apparently the growth represents merely an orderly process of healing which differs from that seen in mammals. Leukocytic phagocytes remove the debris deeper in the tissues, but the epithelial growth fills the defect and effectively holds the uninjured structures together, minimizing the disability and distortion. The epithelium redifferentiates on the surface and provides a cuticular covering with scattered mucus-containing cells.

In view of the apparent absence of fibroblasts in the earthworm, one might question the phylogenetic origin of fibrocytes and fibrocytic collagen. Pauley and Sparks (*9*), though referring to a scar, illustrate in their figure 10, a cellular response to injury in the oyster *Crassostrea gigas*. Pauley, discussing this lesion, indicated a paucity of collagen (*10*). Like the phylogenetic development of cellular and humoral immune mechanisms described by Good and his group (*11*), the phylogenetic development of fibrocytes and fibrocytic collagen may have had a fairly recent origin beyond the invertebrates.

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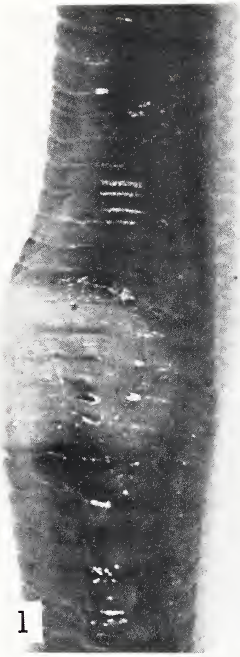
FIGURE 1.—*Note light area* of injury photographed a few seconds after removal of the solid CO_2.

FIGURE 2.—One day after injury the surface epithelium is uninterrupted but distorted, and a thin cuticle can be seen over much of the surface. Immediately underlying the columnar cells several layers of flat, spindle-shaped epithelial cells extend downward into the circular muscle between the broken basement membrane (*arrow* indicates basement membrane on one side). Sarcoplasm of muscle cells is shrunken, and intervening interstitial collagen is swollen and prominent. A few leukocytes were present in the longitudinal muscle layer, just below the lower edge of the photomicrograph. Hematoxylin and eosin. × 230

FIGURE 3.—Also 1 day after injury the down-turned broken basement membrane can be readily seen just above the middle of the mass of epithelial cells that have poured through and displaced the reticular network which characteristically outlines the circular muscle cells. The longitudinal muscle is intact. Immediately underlying the longitudinal muscle is a collection of leukocytes and eleocytes in the coelomic cavity. Edge of the pale chlorogogue tissue is seen on *bottom* with a radial septum at *left*. Reticulum stain. × 150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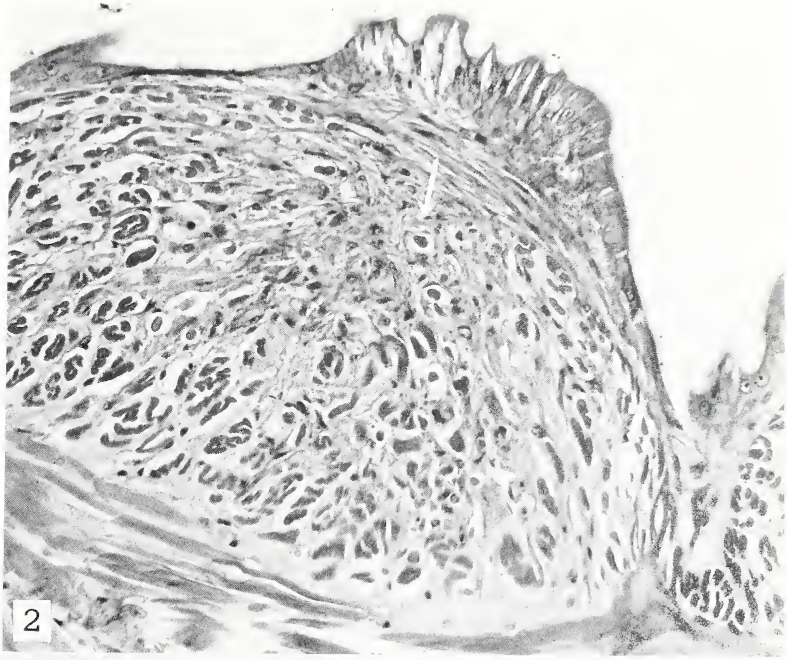
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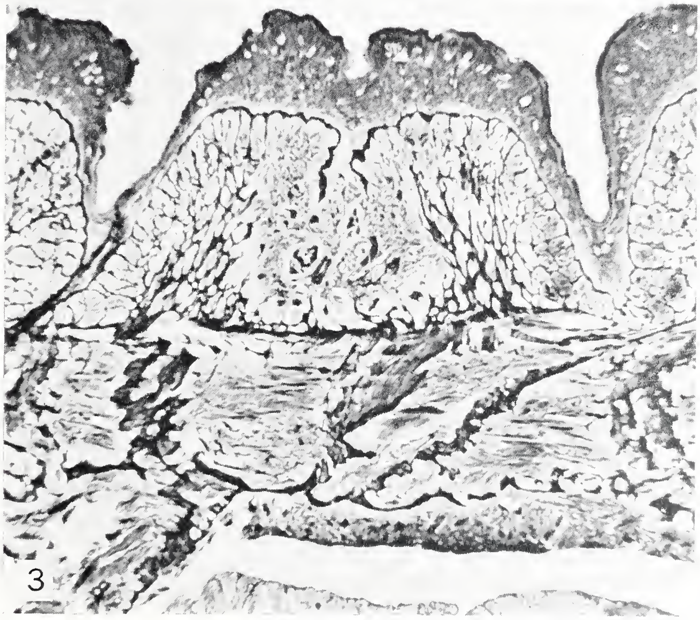
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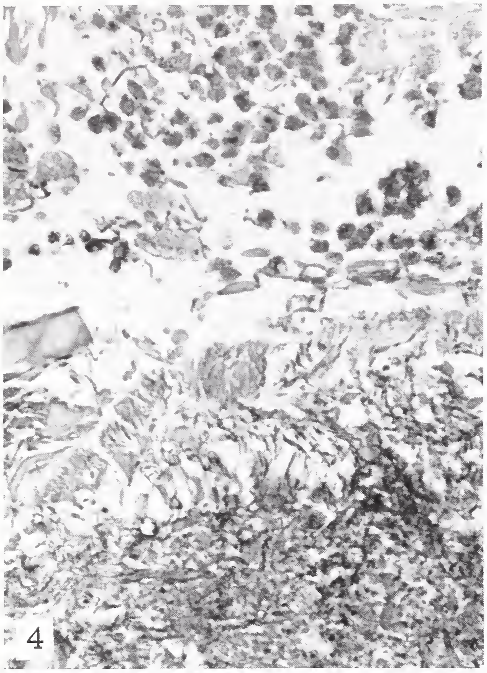
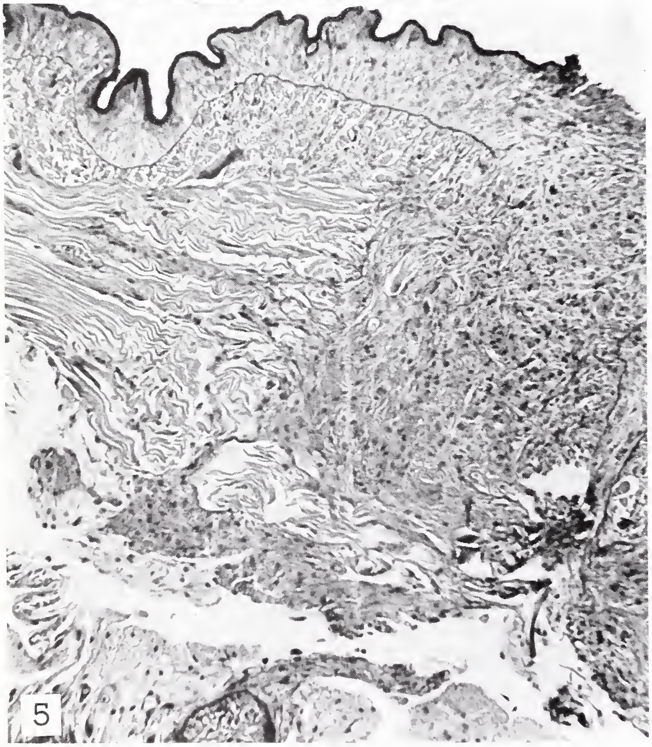


Figure 4.—Edematous deep longitudinal muscle layers contain individual leukocytes with underlying necrotic, fused eleocytes in the coelomic cavity immediately below. Photomicrograph of worm 2 days after injury. Heidenhain stain. × 230

Figure 5.—A mass of rather randomly oriented epithelial cells replaces the upper circular muscle and most of the deeper longitudinal muscle layer. Upper coelomic cavity shows some persisting leukocytic reaction with chlorogogue below. *Note* broken basement membrane and the periodic acid-Schiff (PAS)-positive mucous layer overlying thickened layer of epithelial cells lacking restoration of organized epithelium at *upper right* 14 days after injury. PAS after diastase digestion. × 125



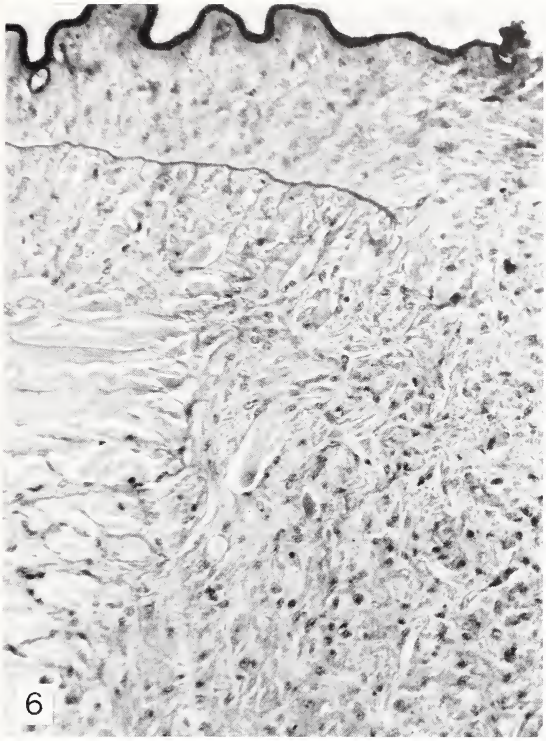


FIGURE 6.—Higher power photomicrograph of the junction of muscle and hyperplastic disorganized epithelial growth showing broken basement membrane. *Note* lack of encapsulation and a few muscle cells trapped in epithelial mass. Stains for collagen reveal almost no collagen in such areas. Periodic acid-Schiff after diastase digestion. × 230

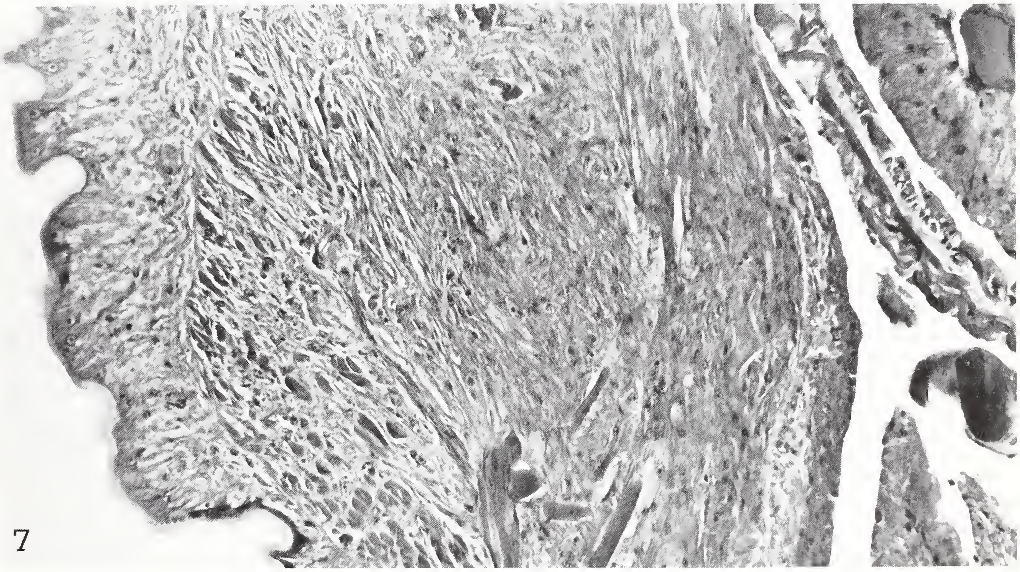


FIGURE 7.—Thirty-one days after injury the epithelial cell mass still persists beneath a restored but thickened surface epithelium. Cells have clearly infiltrated between muscle bundles and line the superior surface of the coelomic cavity. The leukocytes have long since disappeared and the normal chlorogogue and septum is visible. Again special stains reveal little or no collagen in these areas. Hematoxylin and eosin. × 190

Neoplasia and Transplantation Immunity in Annelids[1]

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SUMMARY

This report focuses on the state of knowledge of immune responses in the earthworm. Transplantation immunity to allogeneic and xenogeneic tissue antigens has been successfully demonstrated. However, as autografts have always been permanently accepted, the worm readily distinguishes self from nonself. The second-set phenomenon is characterized by specificity and memory—a single host reacts differently to two different antigens, as indicated by the median survival times of rejected grafts in xenogeneic combinations. Immune theories have been advanced to explain carcinogenesis. Although these theories are derived from exhaustive studies of vertebrates, we believe they could be applied, if only for convenience, to invertebrates. Of these, the earthworm may be a unique species for analyzing neoplastic processes, as these worms have spontaneously occurring myoblastomas which 3-methylcholanthrene (MCA), as carcinogen, could not induce in 24 days. These tumors are histologically similar to benign myoblastomas in humans. MCA crystals placed under autografts produced granulomas, while 1000 r total-body irradiation did not have adverse effects in 40 days. Before more conclusions can be reached, invertebrate oncology and immunity need exhaustive and careful probing.—Nat Cancer Inst Monogr 31: 655–669, 1969.

ONE THEORY to account for the development of cancer in vertebrates depends on an immune response, the graft-versus-host reaction (*1, 2*). According to this theory, carcinogenesis begins with the inactivation or loss of histocompatibility genes of normal cells by either irradiation, virus, or chemical carcinogen. The cell surface correspondingly changes, resulting in the proliferation of now-foreign cells in a normal environment, like cells in a graft-versus-host reaction (*3*). The earthworm system has not been rigorously analyzed to provide such precise evidence as has the prototype mouse system in which tumor or normal tissue transplantation systems were developed largely by Snell (*4*). Yet, there is regularly

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demonstrated histoincompatibility in xenogeneic and allogeneic combinations involving normal tissue transplants in earthworms. The rejection of these antigens, especially in xenografts, is by a primitive immune response characterized by memory and specificity (Cooper, in preparation). Thus, indirect evidence for the presence of histocompatibility genes in annelids may be inferred. As to neoplasia among invertebrates, there is much evidence of, and confusion about, tumors among them (*5*, *6*). Immune reactions in invertebrates are also highly speculative.

This paper presents data concerning invertebrate immunity and neoplasia. I do not suggest that the available information about immunity necessarily conforms to vertebrate explanations. However, a recent symposium included a surge of investigations mainly in the area of serological responses, which indicates interest in features pertinent to humoral immunity. These represent the most up-to-date accounts of invertebrate defense mechanisms (*7–9*). Vertebrate responses only serve as reference points and convenient models for analyzing what may well turn out to be similar processes in both major animal groups once the invertebrates have been exhaustively studied. Alternatively, because of the vast genetic differences and ecological niches between invertebrates and vertebrates, other mechanisms may evolve to accomplish similar tasks.

For heuristic reasons, I review our information on transplantation immunity in oligochaete annelids and present examples of spontaneously occurring cell changes that resemble aberrations and tumors in vertebrates. Since the rejection of experimental foreign tissue antigens provides a situation analogous to the immune mechanism that may have evolved for maintenance and check of spontaneously occurring aberrant cells, we believe that the earthworm could be an excellent species for investigating primitive neoplastic processes.

TRANSPLANTATION IMMUNITY

Most vertebrate animals can reject foreign tissue transplants by an immune mechanism characterized by specificity and anamnesis (*10*). Our recent observations demonstrate that the earthworm possesses a primitive immune response and that specificity and anamnesis are characteristics of annelid immunity to transplantation antigens.

General features of xenogeneic rejection.—When xenografts were exchanged between two annelid species, *Eisenia foetida* and *Lumbricus terrestris*, they regularly healed in like control autografts but eventually underwent rejection at periods ranging from 6–147 days. Gross signs of rejection began as pigment cell breakdown at 4–56 days post grafting. Soon after graft healing occurred, as revealed by histologic analysis, acidophil cells infiltrated the graft-host contact zone. These phagocytes that probably originated from "lymph glands" were more frequently associated with xenografts than autografts. Most second-set xenografts were destroyed by accelerated reactions; however, a significant number developed an opposite effect, *i.e.*, prolonged survival in relation to first-set grafts. Generally, acute rejections (11 days or less) of first-sets always resulted in prolonged survival of second-sets, whereas chronic rejectors brought about accelerated destruction of second-set grafts; short intervals (0–4 days) between first-sets and second-sets led to accelerated rejections, while extended intervals (33–73 days) produced prolonged survival of repeat grafts. Early onset of rejection of second-sets was usually followed by acccelerated breakdown; conversely, late onsets of rejection were followed by slow breakdown (*11*).

Specificity tests.—To rule out the possibility that nonspecific factors play a major role in the rejection of transplanted body wall, we tested the specificity of xenogeneic recognition and rejection. Previous experiments indicated that rejections accelerated when the interval between first-set rejection and repeat grafting was short, *e.g.*, 0–4 days. Moreover, histologic evidence revealed that peak cell response occurred about 5 days post grafting, to produce the following experimental protocol:

1. *Lumbricus* hosts were given first-set *Eisenia* grafts, and 5 days later, second-set *Eisenia* grafts. The results showed that both first-sets and second-sets had essentially the same rejection times but were significantly curtailed over the survival times of single first-sets exchanged between *Eisenia* and *Lumbricus*.

2. The second experiment, and perhaps the most important feature of the specificity test, was the confrontation of *Lumbricus* hosts with first-set *Eisenia* grafts, and 5 days later by second-set *Eisenia* simultaneously with first-set *Allolobophora* grafts (different species but of the same family, Lumbricidae). The *Eisenia* transplants behaved as before, *i.e.*, equal but significantly shortened times of rejection, while the *Allolobophora* grafts were rejected after a longer period, equivalent to the controls of first-set *Allolobophora* alone. These results substantiate that recognition and rejection are, indeed, specific. What is still not ruled out, however, is the possibility that some general or nonspecific reactions, such as those related to inflammation, wound healing, and repair, are also involved in rejection.

"*Self vs. nonself*".—An additional feature of our attempt to solidly demonstrate specificity is one other pertinent experiment designed to determine the specificity of cells reacting differentially to the presence of autografts and xenografts. On *Lumbricus* and *Eisenia* hosts, autografts and xenografts were placed simultaneously in the same graft beds. Both grafts healed in together and apparently existed mutually and compatibly in a contiguous association for a while; however, signs of xenograft rejection always appeared later, followed by complete destruction. The autografts remained permanently and the edge attached to the formerly well-healed xenograft established contact merely with the scar tissue.

Allograft rejection in Lumbricus.—To obviate the assumption that the greater antigenic disparity in xenogeneic combinations was in itself solely responsible for the rejection response which might have been purely biologic, experiments were designed to test the pattern of allograft rejection in earthworms. First-set orthotopic body-wall allografts exchanged between *Lumbricus*, along with autograft controls, healed in promptly; autografts survived permanently. Among worms from Canada and Oregon, intrapopulation allografts in the Canadian worms resulted in 22% permanent survivors, while a comparable Oregon group had 95%; complete rejection occurred in 1% of the Canadian, none in the Oregon group; rejection in the remainder of each group was chronic and incomplete within a maximal observation period of 8½ months. About 5% of reciprocal allografts were destroyed on Oregon hosts and 15.3% on Canadian recipients between 38 and 153 days post grafting. Thus, recognition and specific rejection of tissue alloantigens, although chronic, characterize the primitive immune response of annelid worms.

Allograft rejection in Eisenia.—The allograft response in this genus is apparently different from that of *Lumbricus*. The rejection pattern appears to express a greater degree of antigenic diversity, as revealed by a larger percentage of grafts undergoing total destruction. A cursory examination revealed that during a 7–9 month period approximately 30% of first-set grafts were totally destroyed while there were varying degrees of chronic rejection in 70%. In another group of first-set and second-set transplants, with second transplants performed 5 days after the first transplants, rejection of many repeat grafts was accelerated.

SPONTANEOUSLY OCCURRING PATHOLOGIC PHENOMENA

In this section two pathologic phenomena in vertebrates and annelids will be described. Multinucleate giant cells may be associated with certain inflammatory processes, while myoblastoma, a small benign tumor, is clinically insignificant in humans but becoming of much interest in worms; it is apparently unrelated to the malignant human rhabdomyosarcoma (*12*). Also important is that myoblastomas in our worms occur spontaneously, *i.e.*, they are not induced by either irradiation or other cancer-inducing agents, while multinucleate giant cells apparently occur normally.

Multinucleate giant cells.—Multinucleate giant cells were observed in all worms except in those undergoing degeneration (*13*, *14*). They were oval, slipper-, or dumbbell-shaped with a size range of 14 × 7 μ to 65 × 30 μ. The cell membrane was distinct, surrounding a relatively scanty, granular, acidophilic cytoplasm with vacuoles but there were fewer vacuoles than in coelomic cells. Grouped together in a usually eccentric location in the cells were 10–50 round or oval, small (about 2.5 μ and 2 × 1.5 μ in diameter) nuclei with thick nuclear membranes and evenly distributed chromatin granules. Occasional nuclei were pyknotic. The nucleocytoplasmic ratio ranged from 1:1–1:6, with the smaller cells favoring the lower limit of the range.

Myoblastoma.—In every population some worms had cream-colored swellings or tumors in their body walls, especially those obviously moribund. However, healthier animals usually had single tumors which occasionally regressed. Histologically, myoblastomas consisted of acidophilic, anaplastic cell clusters especially found in the deeper portions of the inner muscular layer, although smaller clusters could also be found in both muscle layers. The cells were polygonal, with a large amount of eosinophilic, finely granular cytoplasm, relatively small, oval, vesicular nuclei with prominent nucleoli and distinct nuclear membranes; cytoplasmic vacuoles were frequently observed. The myoblastoma cell masses were not encapsulated and appeared to extend outward rather than inward. In many instances the cells were lined up along the muscle stroma.

EXPERIMENTALLY INDUCED PATHOLOGIC PHENOMENA

The conditions that were experimentally analyzed can be divided into four major categories: *a*) induction of granuloma formation in xenogeneic tissue transplantation, *b*) fate of allogeneic myoblastoma transplants, *c*) effects of carcinogens, and *d*) lack of irradiation effects. The information pertaining to *c* and *d* are summarized in table 1.

a) *Granulomas:* Many xenografted worms had granulomas in the coelomic cavities, sometimes in proximity to the grafts. Granulomas were large masses of numerous cells and variable amounts of acidophilic, necrotic material with scattered pyknotic nuclei especially in the central regions. Some coelomic acidophils were observed at the periphery of the masses

Table 1.—Effects of MCA and irradiation in *Lumbricus terrestris*

Experimental groups	Results
1. MCA-saturated acetone solution applied every other day for 12 days.	1. Severely affected: sloughing necrotic epithelium and superficial portion of circular muscle. Less severely affected: thick mass of scar tissue. Least severely affected: marked epidermal thickening due to hyperplasia and hypertrophy of secretory cells; increased interstitial cellularity of circular muscle of myoblastoma cell type; slight coelomic cell reaction. Pharynx normal.
2. Acetone applied every other day for 12 days.	2. Essentially the same as 1, but scarring much more extensive, involving even the deeper portion of the circular muscle; no increased interstitial cellularity observed. Pharynx normal.
3. MCA-saturated acetone solution applied every other day for 24 days.	3. Extensive scarring encroaching upon the muscles; moderate to intense cell reaction with some cells invading the inner portion of the longitudinal muscle.
4. Acetone applied every other day for 24 days.	4. Very extensive necrosis involving even the muscles, mainly circular; striking absence of scar tissue; some coelomic cells invading the inner portion of the longitudinal muscle.
5. MCA crystal underneath autograft.	5. Granulomatous mass (histologically like scar tissue with several layers of acidophils on the coelomic side) at graft bed encroaching on the inner portion of the longitudinal muscle.
6. 40 days post irradiation with 1000 r.	6. Essentially normal; no induced myoblastoma and multinucleate giant cells the same as normal controls.

and some lay free in the coelomic cavity or in association with various structures, such as nephridial tubules, peritoneum, chloragogen tissue, or blood vessels. The free cells were small, acidophilic, and polymorphic and had central or eccentric nuclei; nucleoli were rare. Those without nucleoli had obviously clear perinuclear zones of various widths that contrasted strongly with the pink ectoplasmic area and small, purple nucleus. Otherwise, the cytoplasm was homogeneously acidophilic with many pseudopodia. Those with nucleoli did not have clear perinuclear regions. In one instance, the character of the cells seemed greatly changed in a part of the granulomatous mass, *i.e.*, the cytoplasm became highly vacuolated with the vacuoles pushing the nuclei to an extremely eccentric location so that the cells looked fat (signet-ring appearance).

b) *Fate of transplanted myoblastomas:* We have previously determined that the percentage of rejection of allogeneic tissue is very low and the response is chronic in *Lumbricus*, perhaps reflecting a lack of strong alloantigenic differences. Therefore, myoblastomas could be transplanted between worms. Initially both normal grafts and obvious myoblastomas healed, except that edema and pallor were quite pronounced in the myoblastomas, conditions which really never subsided. Preliminary results after 2 weeks indicated that some myoblastomas had been rejected simultaneously with the normal skin, perhaps due to an additive effect. This response differs from that of normal skin only in that normal skin showed no complete acute rejection within 1–2 weeks.

c) *Effects of carcinogens:* After 12- and 24-day periods of every-other-day painting of the body wall with MCA-saturated acetone solution and acetone only, as control, the essential histopathologic features were: necrosis and denudation of the most severely affected areas involving even the underlying muscle(s), formation of scar tissue at the less severely affected parts, marked epidermal thickening at the least affected regions, and moderate to intense coelomic cell reaction. There were few coelomic cells migrating to the muscle; muscle involvement was very slight except for the burning effect of acetone as shown by the severe necrosis of the epithelium and outer muscle layer in the control. In the 12-day MCA group, there were some worms with increased interstitial cellularity of the underlying circular muscle. The interstitial cells were very much like myoblastoma cells. The marked epidermal thickening was due mainly to hypertrophy and hyperplasia of the secretory cells, both mucous and serous. Scar tissue consisted of closely packed cells whose boundaries were difficult to discern, but the interdigitating, spindle-shaped cells could be detected by vertical focus. The scar tissue was acidophilic and rich in long, oval, vesicular nuclei that had distinct nuclear membranes and nucleoli; the cytoplasm was longitudinally striated.

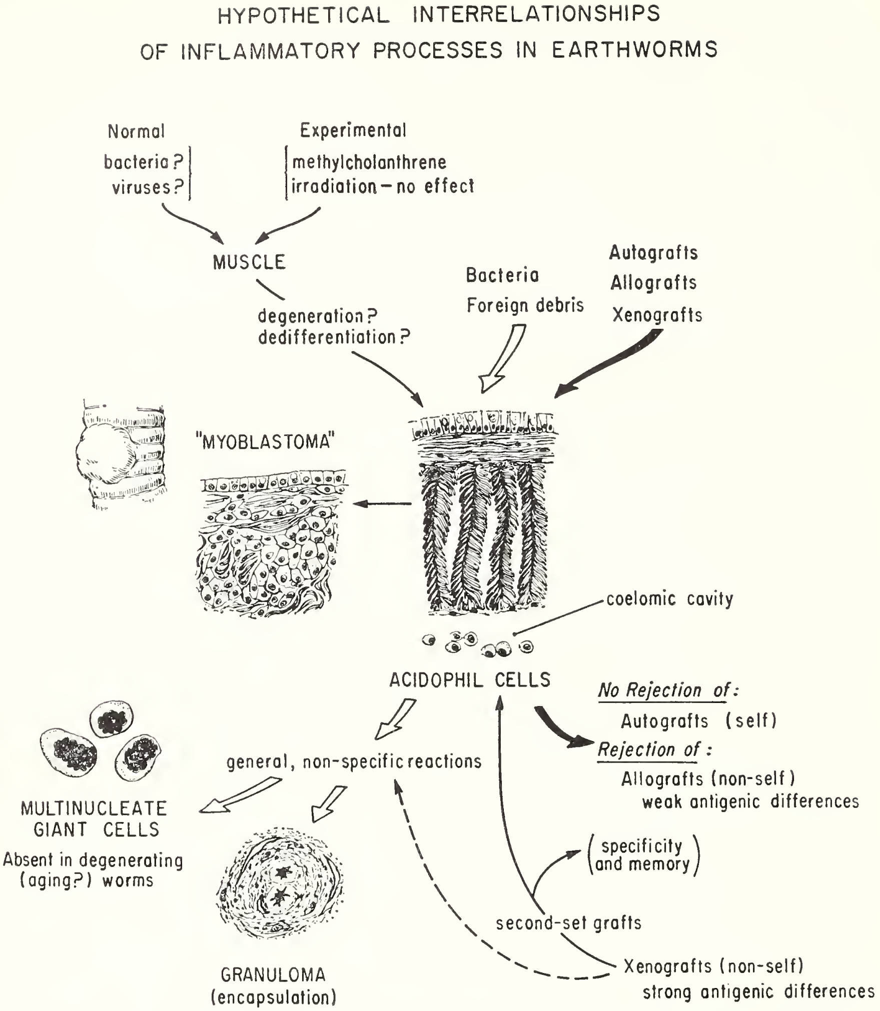
MCA crystals placed under autografts produced granulomatous formations attached to the undersurface of, and encroaching on, the inner portion of the longitudinal muscle. The central region and attached side of the mass looked very much like scar tissue while the coelomic side consisted of several layers of coelomic acidophils.

d) *Lack of irradiation effects:* It has been reported (*14–17*) that myoblastomas and multinucleate giant cells were induced in *Lumbricus* by exposure to 1000 r total-body irradiation. The same amount of irradiation did not produce the same results in our worms. We have routinely observed multinucleate giant cells in close association with obviously normal nephridial tubules, and their essential structure was described earlier in this paper. On the other hand, myoblastomas have been regularly seen in some worms freshly brought in from the wild. Some of these worms were completely riddled with tumors, were generally debilitated, and soon succumbed. Others with as few as one tumor each remained apparently healthy and occasionally the tumors regressed.

DISCUSSION

The essential features of primitive immunity in earthworms to allogeneic and xenogeneic antigens have been presented. In addition, results showed that carcinogenic drugs, like MCA, administered to the integument can lead to tissue changes in the epidermis and both muscle layers. Spontaneous myoblastoma in the earthworm is structurally similar to a benign tumor—a myoblastoma in the human. I also reported on the normal occurrence of multinucleate giant cells of unknown origin. Granulomas were probably induced by foreign material accidentally introduced into the coelomic cavity during grafting. Text-figure 1 shows the hypothetical interrelationships of inflammatory processes in earthworms.

Worms can recognize and reject foreign tissues (xenografts more efficiently than allografts) for allografts are rejected usually in a chronic manner. Specificity is shown by the rejection of xenografts and the acceptance of autografts in both separate and shared graft beds. We believe that if nonspecific reactions were involved, then the contiguity of xenogeneic and autogeneic tissues in shared graft beds might overlap, leading to "nonself" rejection of the autografts as well. Presumably, the cells that homed at the base of both grafts were shared temporarily during the early general nonspecific healing and repair period until immunocompetent cells were differentiated. Memory, though short-lived as evidenced by the fact that short intervals between first-sets and second-sets led to accelerated rejections, while long intervals led to prolonged, chronic breakdown, is another characteristic. That this reaction may be mainly, if not solely, cellular is borne out by the appearance of numerous coelomic acidophils at the graft sites, with the peak of cell response about 5 days post graft. It is more pronounced with xenografts than with autografts, which are always permanently accepted. Since the initial features of this response affect both types of grafts, this reaction is just transient in autografts and probably involved only in the general, nonspecific reactions like wound healing and inflammation and not in the later, more specific response to xenografts.


HYPOTHETICAL INTERRELATIONSHIPS
OF INFLAMMATORY PROCESSES IN EARTHWORMS
Normal
bacteria?
viruses?
Experimental
methylcholanthrene
irradiation – no effect
MUSCLE
degeneration?
dedifferentiation?
Bacteria
Foreign debris
Autografts
Allografts
Xenografts
"MYOBLASTOMA"
coelomic cavity
ACIDOPHIL CELLS
No Rejection of:
Autografts (self)
Rejection of:
Allografts (non-self)
weak antigenic differences
general, non-specific reactions
MULTINUCLEATE
GIANT CELLS
Absent in degenerating
(aging?) worms
specificity
and memory
second-set grafts
Xenografts (non-self)
strong antigenic differences
GRANULOMA
(encapsulation)

Text-figure 1.—Hypothetical interrelationships of inflammatory processes in earthworms.

MCA-saturated acetone solution applied to the body wall caused practically the same, if not milder, necrotizing effects as acetone alone at the severely affected parts—scar formation in less severely affected areas and epidermal thickening, a result of secretory cell hyperplasia and hypertrophy at the least involved areas. Longer periods of treatment resulted in a more intense coelomic cell reaction with some degree of invasion of the muscle, particularly the inner layer. There was increased interstitial cellularity of the circular muscle in some MCA-treated worms, but this was probably a reaction to injury; if it were myoblastoma formation, it was just beginning neoplastic transformation. The interstitial cells were like typical myoblastoma cells of full-blown myoblastomas. These cells are very similar morphologically to human myoblastoma cells, but they are smaller and less chromatic; neither were they pleomorphic (Cooper, in preparation). The etiology and origin of myoblastoma are as mysterious in worms as in humans, so caution must be exercised in identifying the apparent superficial similarity between the two tumors. Many of the cells, when distinguishable normal muscle fibers are still present, are lined up along the muscle stroma. This finding may be reasonably good evidence that these cells do not come entirely, if at all, from transformed muscle fibers.

Presumably, granuloma formation is a means of handling relatively inert foreign materials that are insoluble or cannot be eliminated from the body. Granulomas which were sometimes in proximity to xenografts might have been induced by transfer of such foreign materials as thread fragments from sterile gauze into the coelom during grafting. The handling of these foreign materials resembled Cameron's descriptions of encapsulation in earthworms studied in connection with inflammation (*18*). MCA crystals are very insoluble and apparently inert, since only one caused granuloma formation when placed under autografts. If MCA is carcinogenic in worms, then it might not have been given enough time to exert such an effect.

Except in worms undergoing degeneration, multinucleate giant cells have been routinely encountered and are usually associated with normal nephridial tubules in such a way that the two structures appear as normal constituents of healthy nephridia. Hence we believe that such cells indeed occur normally in these animals. Their formation, although unknown, might be induced by the disposal of wastes. Except that 1000 r apparently sedated the worms soon after irradiation, we observed no adverse effects; there were neither gross nor histologic changes observed 40 days post irradiation. In every respect the multinucleate giant cells resembled those in normal controls. Therefore we believe that irradiation is unnecessary for the induction of multinucleate giant cells and that it seems not to be cancer-inducing at this dose level within the given period.

A plausible analogy between a tissue graft and a neoplasm is suggested; both are foreign in that they are not normal parts of the organism. Theories to explain how an animal handles these two materials are based on immune responses. Typically, the response to a tissue graft by an immunologically competent animal is rejection, but enhancement, which is the opposite reaction and dependent on antibody (*19*), is also possible. Presumably, neoplasms are similarly taken care of and when enhancement does occur a tumor grows because, paradoxically, it escapes the confines of the typical immune system and may operate autonomously. Although we have no information on the presence of antibodies to normal and/or tumor antigens in worms, second-set normal xenografts are mostly accelerated while many are prolonged (enhanced?).

The evidence of primitive immune competence in earthworms suggests that the evolution of immunity with components similar to those in classic vertebrate responses may not have originated in vertebrates, specifically in the cyclostome fishes (lampreys), as had been advanced previously (*20*).

Moreover, the recent rigorously studied graft rejection responses in hagfishes offer more convincing evidence that adaptive immunity did not appear initially with lampreys (Hildemann, personal communication). A hypothesis was proposed that the thymus, a lymphoid organ, is necessary to development of the immune system. Moreover, the thymus evolved as a mechanism for facilitating tumor cell recognition, regression, and disposal. In addition, adaptive immunity may not have evolved in invertebrates and the lowest vertebrates, in which the threat of aberrant cell development seemed low. Rather, the "higher" vertebrates depend on immune competence for survival, since cellular differentiation is much more complex and malignant potential greatly increased (*21*, *22*).

Evolution of adaptive immunity may be more complex since invertebrates, like other living creatures, require a system of defense to survive. Invertebrates lodge in hostile environments and are constantly vulnerable to potentially fatal pathogens. Moreover, it appears unlikely that adaptive immune responses appeared simultaneously with the evolution of the first vertebrates; invertebrates undoubtedly have a built-in system capable of distinguishing "self" from "nonself" that should be highly specific; this can be inferred without experimental design. Otherwise they would probably disappear after destroying themselves by nonspecific reactions in attempts to do away with harmful pathogens. Burnet (*22*) suggested, in regard to ameba-phagocytosing microorganisms, that by some mechanism the ameba can distinguish between the chemical structure characteristic of "self" and other different chemical structures which are recognized as "nonself."

I propose, therefore, that food-getting and defense are indistinguishable features of phagocytosis in "lower" invertebrates like the protozoa. Among "higher" invertebrates, as suggested by our studies of primitive transplantation immunity, phagocytosis, which may be a major feature of graft rejection, is primarily a defense mechanism and is specific but it may function independently of macromolecules unlike vertebrate immunoglobulins. Moreover, humoral immunity characterized by antibodies to soluble or particulate antigens may not be present. Among the vertebrates also are transplantation immune responses and delayed hypersensitivity; the evolution of a newer cell type, the plasma cell, capable of producing immunoglobulin may have originated initially among the "lower" vertebrates.

Generalizations about the phylogenesis of immunity depend upon accepting the view that the thymus is necessary for differentiation of immunity. Therefore, an animal without a thymus would be expected to be without an immune system. The presence of all other functional systems in all invertebrates is undeniable, as demonstrated for example by the recent scholarly treatise by Bullock and Horridge (*23*) on invertebrate neurophysiology. Thus, as primitive immunity among earthworms is recognized, a thymus analogue becomes necessary. It is significant and perhaps relevant for evolutionary perspective that the earthworm's phagocytic organs ("lymph glands") are contiguous with the digestive system (*24*), and may have been derived developmentally from endoderm like the vertebrate thymus.

Cellular differentiation may not be much more complex in higher forms. However, countless examples of cell specialization and differentiation among invertebrates are recorded (*25*). With this specialization, invertebrate cells probably could, by mutation, become aberrant or neoplastic as vertebrate cells do. If these cells can go off into other avenues, then the lack of genetic stability could frequently lead to harmful directions or neoplastic transformations. A good case in point relative to the plasticity of invertebrate cells is the totipotentiality of neoblasts, cells involved in regeneration in flatworms and annelids (*26*). Thus, following the currently accepted line of reasoning, we may suggest that since invertebrate cells can become neoplastic, invertebrates must possess an immune system—or, since invertebrates have a primitive immune system, they could develop neoplasms.

It is crucial that a need for a defense mechanism against foreign materials evolved, and each animal group solved the threat of infectious extinction by diverse types of immune systems. Hence particular environmental factors should be considered. Poikilothermic vertebrates give immune responses dependent on temperature and a slightly different array of lymphoid organs from homiotherms. In anuran amphibians, for example, the larvae which are aquatic and are developmentally immature, are capable, very soon after hatching, of mounting an immune response to both transplantation and soluble antigens; they are immunologically competent with lymphoid organs hooked by vascular connections to the gills (*27–29*).

Mayr (*30*) believed that much could be learned about the evolution of immunologic defense mechanisms if the purely genealogical approach were supplemented by one which emphasizes the adaptive significance of these mechanisms. Instead of referring to a "better" mechanism associated with "higher" vertebrates, he suggested asking what kind of mechanism or system of immune responses would be expected from animals adapted for specialized ecologies. For example, are there consistent differences between freshwater and marine cyclostomes, between freshwater and marine teleosts? Or are the high levels of urea in the blood of elasmobranchs related to the differences in immunological responses of sharks and marine teleosts? Does homiothermy require a different system from poikilothermy?

Hildemann is optimistic about the existence of immunity in invertebrates, stating essentially that specific immunologic competence demonstrable in cellular and/or humoral responses probably exists in Eumetazoan invertebrates, or that immunologic phylogenesis is revealed in progressive differentiation and specialization of functions through the vertebrate series to mammals. Furthermore, with the profound differences in genetic and physiologic endowments among phyla ranging from invertebrates to mammals, pathways of immune responsiveness substantially different from the familiar mammalian norms might well be found in invertebrates (*31*).

It seems reasonable to conclude that invertebrates do possess immune systems, although the nature of the mechanisms is decidedly unknown. Reactions may be as numerous as the varied taxonomic groups, as is true of most rigorously studied vertebrates. Invertebrate cellular immunity may be closer to vertebrate reactions and may represent the more primitive responses. In the absence of classic vertebrate-type immunoglobulin in invertebrates, a real dichotomy would be evident in the evolution of immune responses. On the other hand, immunoglobulin precursors may be present.

Our knowledge of neoplastic processes in invertebrates is also confused and understanding of simpler mechanisms could be fruitful. The two broad areas of invertebrate oncology and immunity must be exhaustively investigated before accurate conclusions can be made.

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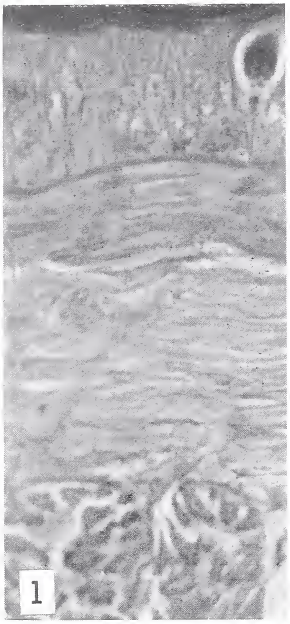
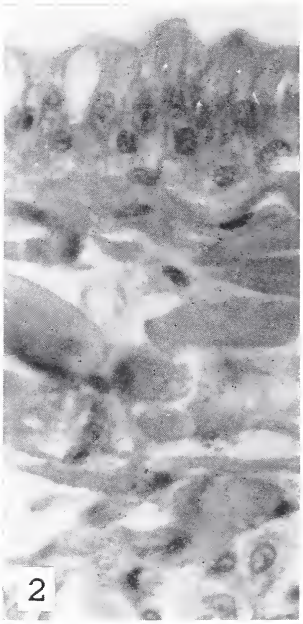




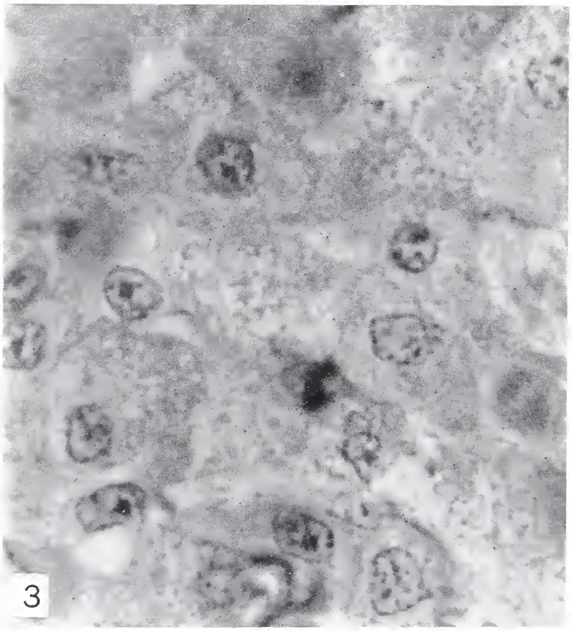
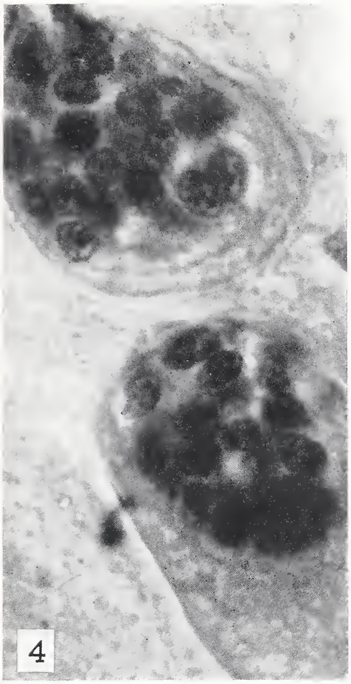
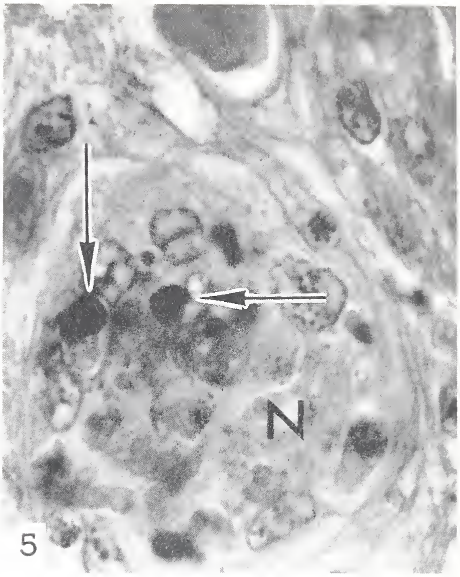


FIGURE 1.—Normal body wall of earthworm consisting of a pseudostratified, cuticular, columnar epithelium, an outer circular muscle layer and an inner longitudinal muscle layer, and (not shown) a simple squamous epithelium, the peritoneum. Hematoxylin and eosin. × 830

FIGURE 2.—Integument of earthworm with myoblastoma shows less orderly arrangement of epithelial cells and the hardly discernible basement membrane and cuticular layer. *Note* also irregularity of base of epithelium. The regularity of the muscle-fiber arrangement has been disrupted by increased interstitial cellularity. Hematoxylin and eosin. × 830

FIGURE 3.—"Myoblastoma" cells of earthworm. *Note* polymorphic cells with large amount of cytoplasm and small, not very chromatic nuclei with nucleoli. Hematoxylin and eosin. × 1000





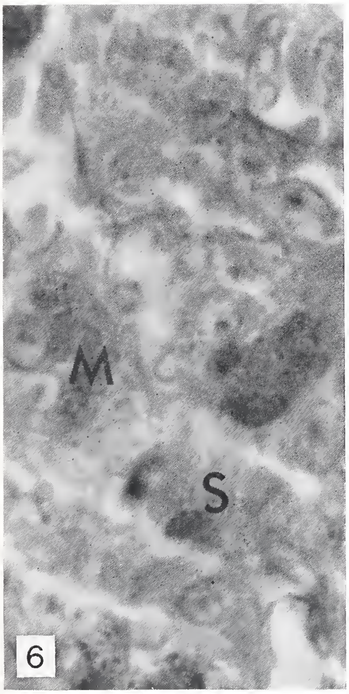


FIGURE 4.—Multinucleate giant cells of earthworms. *Note* very thick cell membranes with relatively small amount of cytoplasm and numerous, small, round or oval, dark-staining nuclei grouped together at eccentric locations in the cells. Hematoxylin and eosin. × 1000

FIGURE 5.—Granulomas of earthworm. *Note* area of necrosis, N, with some pyknotic nuclei (*arrows*) and whorled, spindle-shaped cells at periphery. The latter are very much like scar tissue cells. Hematoxylin and eosin. × 1000

FIGURE 6.—Basal portion of markedly thickened epithelium after application of methylcholanthrene-saturated acetone solution. These are secretory cells; those with coarse granules, M, are mucus-secreting cells, while the finely granular, S, are serous cells. Periodic acid-Schiff. × 1000

Review of Tumors and Tumor-Like Conditions in Protozoa, Coelenterata, Platyhelminthes, Annelida, Sipunculida, and Arthropoda, Excluding Insects[1]

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INVERTEBRATE GROUPS in which the overwhelming majority of tumors have been reported are reviewed elsewhere in this volume, so I will review briefly the status of knowledge of neoplasia in the remaining major invertebrate taxa not discussed at the Symposium. Aside from the insects, most reports of tumors in invertebrates are in the mollusks, with other groups variously represented and some entire phyla unreported. We are uncertain whether some groups, such as the mollusks, are more inclined toward neoplasia than others or whether they are simply more frequently studied by biologists, and their tumors more readily observed. As Scharrer and Lochhead (*23*) pointed out, many serious difficulties are encountered in any attempt to assess the relative abundance and mechanisms of tumorous changes in invertebrates. Much past difficulty resulted from the fact that most zoologists familiar with gross and microscopic anatomy of invertebrates are inexperienced in tumor diagnosis and, on the other hand, pathologists specializing in tumor research with vertebrates are generally unfamiliar with anatomy, physiology, and reactions to injury of invertebrate animals. Recent events, particularly this Symposium, show this to be a diminishing problem. However, except possibly in the insects, tumors in invertebrates have not been sought or studied as extensively as in vertebrates.

This review will not attempt to define or establish criteria for neoplasia in the invertebrates, although many structures described as tumors in the literature are clearly hyperplasias or unusual proliferations of typical cellular components in response to injury or parasitic invasion and would not be considered tumors by the concepts developed in pathology of vertebrates. Points of difference that permit distinction between neoplastic and non-neoplastic changes are perhaps debatable and require experimental study not usually possible with the small numbers of tumors recognized among invertebrates in nature.

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[1] Contribution No. 303 from the College of Fisheries, University of Washington.

PHYLUM PROTOZOA

Opalinid nuclear abnormalities.—Metcalf (*17*) reported on several instances of abnormal specimens of Opalinids, a group of parasitic ciliates inhabiting the large intestines of frogs and toads. These opalinids, *Opalina abtaigona*, contained, in addition to the typical numerous normal nuclei, many abnormal nuclei which Metcalf thought closely resembled certain cancerous cells in mammals. Among the diagnostic features of mammalian cancers are multipolar and other atypical mitoses, amitotic division of chromatin, degenerating nuclei, and enlarged cells. Metcalf observed nuclei with an arrangement of chromatin into a coarse-meshed cap on a clear, glassy-appearing body, and of the granular achromatic substance into well-developed and fairly regular lines of granules resembling a mitotic spindle. The chromatin material, however, never formed distinct chromosomes. The chromatin-capped spheres were usually single, but two or three such masses of chromatin were sometimes found in a single abnormal nucleus.

All these phenomena, in Metcalf's opinion, indicated amitotic division of the chromatin. The division of the chromatin was often into unequal masses, and subsequently stages of nuclear degeneration resulted in gradual disappearance of the nuclear contents and wall. The chromatin-capped spheres and chromatin masses were the most persistent structures, but ultimately disappeared, leaving empty spaces in the cytoplasm, often with remnants of chromatin masses. Metcalf further noted that the degenerating nuclei were larger than normal, again resembling nuclei of some neoplasms.

Additional study of other opalinids indicated, in Metcalf's opinion, that the abnormal nuclei were derived from cells in which amitosis followed abnormal mitosis. In *Protoopalina caudata*, which normally contains two nuclei, he reported occasional cells whose nuclei divided without cytoplasmic division; others contained nuclei whose chromosomes had divided to produce double the normal complement without either nucleus or cytoplasm dividing. Metcalf noted that in all the abnormalities, cells and nuclei were greatly enlarged and nuclear degeneration and disappearance also occurred occasionally along with degeneration of cytoplasm in the enlarged cells. He summarized those abnormal characteristics in opalinids which he believed corresponded cytologically to mammalian cancer as: "1) enlarged cells with enlarged nuclei arising by division of the nucleus or by fusion of nuclei. Such nuclei have too many chromosomes; 2) unequal division of such enlarged nuclei causing still further distortions in kinds as well as numbers of chromosomes present; 3) amitotic division of the chromatin without division of the nucleus; 4) degeneration of nucleus and of cell."

Many, if not most, modern workers tend to discount Metcalf's findings simply as examples of abnormal mitoses. The characteristics he described do resemble those frequently associated with neoplasia in higher animals, and some of the mechanisms concerned may quite possibly be similar. Metcalf's report is better understood when viewed in the perspective of his own time, for he was stimulated to fit his observations to the theory of Boveri (*1, 2*). This theory in turn was based on the observation of unequal nuclear division following polyspermic fertilization of sea urchin eggs, coupled with the demonstration still earlier by von Hansemann (*31*) that unequal division of chromatin occurred in human neoplasms.

Mottram (*18*) demonstrated that benzo[*a*]pyrene, which increases cell growth when applied to vertebrate tissues, increased growth rates in *Paramecium* cultured in various concentrations of the substance. Paramecia of abnormal shape developed in concentrations of 1 and 0.5 parts per million after prolonged culture (122 and 62 days).

The experimental cultures never underwent conjugation, and abnormal specimens removed and placed in normal media as single-cell cultures developed into populations of abnormal paramecia. Some were many times normal size, some were midgets, others were "Siamese twins" or triplets, and some were apparently normal. Offspring of the abnormal forms were often normal and outgrew the abnormal, but some abnormal forms bred true through many generations. Long exposure to the carcinogen, therefore, results in the production of polymorphic cells in a striking similarity to that of mammalian tumors initiated by benzo[*a*]pyrene in that they occur among cells long stimulated to accelerated growth; they occur only after long exposure to the hydrocarbon; the abnormal cells continue to be produced after the stimulus is removed, and the cell population is highly polymorphic.

PHYLUM PORIFERA

I know of no reports of tumors or related neoplastic growths in the sponges. It is not known whether this is because the group lacks such processes or because investigators have not sought, recognized, or reported neoplastic abnormalities in the sponges.

PHYLUM COELENTERATA

Reports of neoplasms in coelenterates are infrequent in the literature, and their authenticity has often been questioned. Korschelt (*14*) reported strange "neoplasms" rather than normal buds in the polyps of the hybrid *Syncorybe decipiens* during senescence.

Squires (*26*) described three abnormal corallites from the only known specimen of *Madrepora kauaiensis*. Since the specimen was dried and the polyp tissue destroyed before Squires' examination, soft tissues could not be studied and only abnormalities of the skeleton could be described. As coral growth is accretionary and developmental sequences are preserved in the structure of the skeleton, more light is shed on the probable structure of the soft tissues in the corals by study of the skeleton than in most invertebrate groups.

Although malformed corals are relatively common, most abnormalities can be directly attributed to the effects of predation or reaction to injury. Those not readily explainable by these phenomena have generally been called abnormal and not studied further. In the original description of *M. kauaiensis*, at least one unusual corallite had been noted and interpreted as an individual of another species growing attached to *M. kauaiensis*. Squires disagreed with that interpretation because the skeletal elements of the abnormal corallites were organically connected to the colony and hence were formed asexually by polyps lower on the branch. The fact that the abnormal polyps were conspicuously larger than their "siblings" suggested a greater growth rate, an impression supported by structural features indicating rapid growth.

Madrepora characteristically possess solid, thick septa with minutely dentate margins and sides. The pathologic corallites had highly fenestrate and lacerate septa, a condition characteristic of mussid corals and one that provided the basis for the suggestion, in the original description, that a second species was present.

The skeletal arrangement of the abnormal polyps suggested that the normal division and insertion of the mesenteries were followed until the normal polyp size was exceeded, and then mesentery formation and septal insertion became disordered and chaotic, destroying the normal symmetry of the polyp. The pathologic polyps arose from normal individuals, which also (earlier) gave rise to normal polyps by the usual asexual process.

Squires noted that any unusual environmental condition initiating the abnormal growth should have affected more than 3 of the 239 polyps in the colony, but that other polyps of similar age were unaffected. Although corals are preyed upon by fish and predaceous invertebrates, the regenerative capabilities are high and repair is relatively rapid and complete. Occasional duplication of septa along the margins occurs but results from duplication of only a single mesenteral pair. Since the disturbance of growth in an injured coral is minimal, Squires did not think the abnormal corallite resulted from predation or accidental injury, chiefly because the polyps were unusually large. He believed that the natural growth-regulating mechanism was removed, allowing the abnormal increase in size. Although the actual nature of the growth-regulating mechanism in corals is not known, it is thought to be related to the attainment of sexual maturity, because growth rate decreases sharply after sexual maturity is reached.

White (*30*) and Soule (*25*) both took issue with Squires' interpretation. White agreed that the growths were neoplasms by the general definition of aberrant new growth, but warned against the possible inference that they represented malignant neoplasms or cancers. He noted that a malignant neoplasm usually arises originally as an individual altered cell which gives rise to additional abnormal cells resulting in a malignant growth, and that generation of normal polyps by all three of the abnormal corallites would be unlikely. White offered an alternative theory as to their origin by pointing out the extraordinary resemblances in structure and distribution to the galls, common on plants, resulting from various sedentary predators. These occur in aquatic environments, *i.e.*, on the green algae *Vaucheria* as a result of the presence of a rotifer. The abnormal growth resulting in galls is initiated by "growth hormones" secreted by the resident predator.

Soule (*25*) believed that the abnormal corallites were actually colonies of a cyclostomate ectoproct belonging to the genus *Tichenopora*, which looks so much like the mineralized portions of *Madrepora* that many species of the genus were originally described in the genus *Madrepora.*

Squires (*27*) agreed with White that gall formation probably occurs in the corals because they are hosts to numerous organisms causing galls in other organisms, but believed that the abnormal corallites originated from other stimuli, since examination of the specimens gave no evidence of coral occupancy by other organisms. In reply to Soule's assumption that the growths were bryozoan colonies, Squires noted that, although he had no certain *Tichenopora* to study, X-ray diffraction patterns differ in bryozoans and corals, and the diffraction patterns in the abnormal corallites resembled those of normal corallites from other portions of the colony.

PHYLUM PLATYHELMINTHES

Tumors among the flatworms have been reported only in the class Turbellaria. This is most probably related to the high regenerative capacity of the turbellarians, either through a presumed intrinsic potential for neoplastic growths when the regenerative processes are misdirected or because the multiplicity of investigations of planarian regeneration has increased the probability of observation of both spontaneous and experimentally produced tumors. Dubois (*4*) demonstrated that regeneration in planarians is accomplished through the activities of undifferentiated totipotent cells, the neoblasts. When the wound occurs, surrounding neoblasts quickly differentiate to form new epidermis, while other neoblasts migrate to the wound area and differentiate into the other cell types necessary to replace the damaged or lost tissue.

Several workers (*7*, *15*, *28*) have reported spontaneous tumors in planarians. In apparently the first record of such an occurrence, Goldsmith (*7*) observed a specimen of *Dugesia tigrina*, in a stock culture maintained in his laboratory for 3 years, with two dorsal pigment stripes rather than the typical one. Detailed examination revealed that the abnormal animal possessed two pharynges. Following isolation, the planarian developed a small outgrowth from the dorsal surface of the postpharyngeal region after 4 weeks, and two additional outgrowths appeared within 2 months. Attempts to produce a clone from this animal, by making a transverse cut anterior to the pharynges in the abnormal animal and in subsequent planarians derived from the operation, resulted in the production of eight animals at the time of reporting. Interestingly, the transverse cuts were not followed by the typical regeneration of a single head at the anterior end of the posterior piece and a tail at the posterior end of the anterior piece. Instead, multiple heads, as many as eight in one instance, with vesicles and pigment cups and multiple tails developed, similar to those produced by the splitting operation in previous regeneration studies. Also, an additional dorsal outgrowth, produced by the transverse cuts, developed in one animal. Although no figures were presented and their histology was not discussed, Goldsmith noted that the outgrowths resembled those produced experimentally by a variety of stimuli which will be discussed. Stephan (*28*) described apparently similar spontaneous tumors in the same species. Again, no figures were included, but the author presented a description of the tumors' histology, noting that they appeared as massive infiltrations of the parenchyma by glandular cells. Secretions accumulated in open pockets developing in the base of the dorsal outgrowths. The rupture of the pockets or pouches, followed by a disintegration of the crown, produced on the dorsal surface an appearance identical to that of the ventral surface of the worm.

Lange (*15*), working with colonies of *D. etrusca* and *D. ilvana*, found high incidences of spontaneous tumor development in both species. Fifty-four tumor-bearing animals were studied and, since no discernible difference between the tumors of the two species was observed, they were described together.

Most tumors originated as reddened swellings or lumps, or groups of lumps, on the posterior tip of the animals, stretching the epidermis and distorting the tails. As the growth slowly developed, tail thickness increased greatly impeding movement, and the tumors became darkly pigmented and eventually developed clear, white tubes from which mucus was occasionally extruded.

The tumor-bearing animals continued to divide, even after the tumor became the largest part of the body, with the anterior giving rise to normal individuals in which no tumors were observed over a 1-year period, while the posterior portions retained the tumors. The abnormal posteriors regenerated new heads at normal rates, then redivided, leaving a smaller posterior portion still bearing the tumor. Eventually the posterior portion and the tumor lysed.

Only one specimen, a *D. etrusca*, developed tumors on both the anterior and posterior region. Fission did not take place in this animal and the midbody shrank rapidly until lysis of both the unaffected portion and the tumorous anterior and posterior regions occurred.

Wound repair and regeneration were abnormal in the tumors. A cut in normal tissue results in muscular contraction, which pinches off the wounded area and prevents cellular loss to the exterior. This is followed by differentiation of local neoblasts to form a new epithelium over the wounded surface, and finally by immigration of neoblasts into the area from distant nests of neoblasts to regenerate the missing or damaged tissues.

In contrast, incisions into tumorous tissue indicated no muscular contraction. The wound remained open, with cells streaming out of the body until all the tumorous tissue and large quantities of normal tissue were lost. Eventually contraction did occur, but well beyond the original boundary of the tumor, and normal regeneration followed.

When Lange reduced the food to one feeding per week in his tumorous populations, no new tumors developed except in two worms in which tumors first appeared 1 week after feeding had been reduced.

Although most tumors originated at the posterior tip of the worms, in two instances a tumor developed in the dorsal midregion just behind the mouth. The tumor in each animal assumed a wide, crater-like appearance with a clear ring of tissue forming a rim around the apex of the mound. Neither of these animals divided, and additional tumors developed on the posterior tips of the animals. The latter tumors began to lyse and the animals were fixed for histological study.

Histologically, tumor-bearing animals exhibited increased tail thickness, consisting mainly of papillary and cryptlike structures covered and lined by mucus-producing epithelium and a broken or perforated epithelium. The crater-like tumors arising anteriorly contained large masses of highly differentiated tissue, including nervous, parenchymal, and epithelial tissues, and crypts lined with epithelium. The top of the tumor was lined with an epithelium resembling that of the ventral body surface, as were the epithelium-lined crypts, and large pockets of neoblasts were scattered around the tumor. Lange postulated that the ventral type of epithelium lining the tumor crater may have resulted from the bursting of a crypt. He further stated that the cells and tissues comprising the tumors were not in themselves abnormal, but that their relationship to surrounding tissues caused the abnormality.

Foster (*6*) reported nodular growths in *D. dorotocephala* resulting from treatment with the carcinogens benzo[*a*]pyrene, 3-methyl-4-dimethylaminoazobenzene, and benz[*a*]anthracene. Her work is reviewed along with the presentation of new results in another paper in this volume.

PHYLUM ANNELIDA

Reports of tumors in annelids are relatively rare and confusing. Thomas (*29*) studied large numbers of polychaetes, *Nereis diversicolor*, that possessed globular, whitish, tumor-like structures at various sites, though predominantly on the dorsal or ventral surface of the middle third of the body. Microscopically, the growths were seen to develop either around degenerating oocytes or degenerating bristles. Thomas was aware of the possible error in designating them tumors, since they developed as a result of irritation provoked by the degeneration of the oocytes and bristles. He contended, however, that voluminous amounts of new tissues were formed and, therefore, they were neoplastic structures for which the term "tumor" was completely appropriate.

PHYLUM SIPUNCULIDA

Tumors have been reported on two occasions in *Sipunculus nudus.* Herubel (*13*) described a small tumor on the side of a specimen of this species. He was particularly interested in the abnormality because it was the first observed among the more than 1,000 sipunculids he had dissected over a number of years at the Roscoff Laboratory. The tumor was on the right posterior third of the animal and consisted of an oval base approximately 1 cm in diameter and an elevated hump projecting outward approximately 5 mm. The tumor consisted of numerous cells which Herubel described as compressed into a hollow cavity within the skin, limited on the top by the epidermis and cuticle and on the bottom by the circular muscles of the integument. Careful microscopic study of sectioned material convinced Herubel that the muscle fibers were degenerating, leading him to diagnose the structure as a muscle tumor. He did not attempt to ascertain the cause of the condition, but assumed it was of parasitic origin. The cells in the mass were, according to the author, unquestionably amebocytes or leukocytes carried there by the cutaneous canals. He further noted that some amebocytes in the section were in the process of phagocytizing degenerating muscle fibers.

Unfortunately, Herubel presented no illustrations, so it is impossible to determine whether the abnormality actually represented a tumor or, as seems much more likely from his own description, a heavy leukocytic response to an injury. Herubel's description of a hollow cavity filled with cells is a more convincing description of an abscess than a tumor.

Ladreyt (*16*) described a tumor that he considered malignant in the dorsal canal of Poli in *S. nudus.* A chance dissection revealed the neoplasm originating from the endothelium of the anterior third of the eosophageal tube and appearing, at the opening of the coelom, like a large, pinkish-gray pea projecting into the coelomic cavity. Normally the cells covering the internal and external surfaces of the canals of Poli are rather flattened and closely resemble the endothelial cells of the lymphatic system of vertebrates. Like the peritoneum which it resembles, the remainder of this vascular epithelium contains ciliated and nonciliated cells which differentiate into the urn cells, the red blood corpuscles, and the chloragogen cells. In the tumor-bearing sipunculid, all the cells of the vascular endothelium were modified at the beginning of their neoplastic evolution. Ladreyt stated that this observation demonstrates the generality of the phenomenon of dedifferentiation, which supposedly characterizes the beginning of the cancerous process in the higher vertebrates. This "precancerous" stage in the sipunculid was, according to Ladreyt, indicated by marked hypertrophy, amitosis, frequent multipolar mitoses, and nuclear fragmentation in cells in the walls of the canal of Poli.

The cells were thought to have developed progressively into fusiform or epithelioid variants, so that the fully transformed atypical cancer cells were greatly elongated, with free extremities more or less swollen. They contained large nuclei, irregular or globular in shape, that were rich in chromatin. The tumor consisted of a massive aggregate of one stratum or several strata of these cells, greatly elongated in the anterior-posterior direction and surrounding the vascular axis.

Ladreyt noted that the functional capacity of the endothelium appeared closely related to the degree of morphological dedifferentiation. He believed the endothelium was functional in the early stages of this change, but progressively lost function so that no traces of respiratory pigment or excretory activity remain in the atypical cells.

Ladreyt posed the question of whether this growth was a cancerous tumor and, if so, to what histological type it was related. He noted that hyperplasias associated with inflammation are common in invertebrates and are most frequently granulomas of parasitic origin, quite obviously different from the tumor in question. In concluding that the tumor was cancerous and malignant, he listed the following supportive points. 1) There was a precancerous stage characterized by morphophysiological dedifferentiation and very intense multiplication of the parietal cells, resembling precancerous stages in the digestive tube of higher vertebrates. 2) A cancerous stage followed, in which the endothelial elements were clearly atypical, with the malignant cells crowding into the general body cavity and its appendages (cutaneous canals). Its malignant nature was indicated by the tumor's effect on the respiratory and excretory functions and by regression and degeneration of the muscular and nervous systems. The morphology, development, and effects on the organism seemed to Ladreyt so similar to the comparable properties of endotheliomas and epitheliomas in higher animals that he considered it a complex malignant tumor which he called an endoepithelioma.

Other authors have not considered the evidence sufficient for a positive diagnosis of malignancy and, as Scharrer and Lochhead (*23*) pointed out, the lack of photomicrographs or any illustrations of the tumor makes conclusive interpretations difficult, if not impossible. I cannot help wondering how Ladreyt could ascertain so much about the origin and development of the tumor when only one example, and that apparently well-developed, was available for study.

PHYLUM ARTHROPODA (EXCLUSIVE OF INSECTS)

So much has been written about tumors of insects, especially of fruit flies (*Drosophila*) that one might expect high tumor incidence in the remaining classes of the phylum. This, however, is apparently not true, judging from the paucity of reports of neoplasms in noninsect arthropods.

I do not propose to discuss tumors in insects, but several reviews of this subject are available, the latest by Harshbarger and Taylor (*9*). Their review was directed primarily toward the literature published since 1950 and they noted that, of the more than 400 papers on tumors in insects, about 80% are concerned with *Drosophila.*

In contrast to this voluminous literature on the neoplasms of insects, I could find only 8 papers concerned with tumors or tumor-like growths in the remaining arthropod groups. Several of these, as will be seen shortly, are of dubious validity or are only briefly mentioned in papers primarily concerned with other information.

A tumor-like structure was reported in the primitive arthropod, *Limulus*, by Hanström (*8*). It appeared as a chitinous foreign body near the anterior end of the brain, with a portion of the outer skin between the anterior margin of the carapace and the oral appendages. This structure, as pointed out by Hanström, resembled "dermoid" cysts of man and other vertebrates in that it was formed by recession of a saclike portion of the ectoderm within the body proper, where it continued to secrete successive chitin layers.

In the class Crustacea, tumors have been reported in lobsters, a shrimp, and a crab. Herrick (*11*, *12*) briefly described a large growth on the side of the carapace of a lobster caught in Vineyard Sound, Massachusetts. A crater-like depression was covered by a membrane over the top of the growth. Herrick thought the growth was a "sore" resulting from a wound that had failed to heal. He noted that a similar case had been mentioned by Rathbun (*21*), and that Prince (*20*) included a description of a tumor-like growth originating in the wall of the stomach sac of a large lobster dying after the growth perforated the carapace. Although I have not seen the latter report, apparently no gross photograph, histological studies, or photomicrographs were made of either Rathbun's or Prince's cases. This seems particularly unfortunate in regard to the growth arising in the stomach sac.

Savant and Kewalramani (*22*), in recording a new host record of the isopod parasite *Bopyrus squillarium*, stated that the parasite caused a 7 mm tumor in the left branchial chamber of the host shrimp, *Paloemon tenuipes*. Since this genus of isopods typically causes a pronounced swelling on the side of the cephalothorax through the presence of the female in the host's gill chambers, it is suspected that the authors used the term tumor in its broadest sense and were emphasizing the size of the swelling rather than suggesting neoplasia.

There is no question, however, of Fischer's (*5*) intentions in his description of tissue which he stated resembled cancer in the abdomen of a crab, *Carcinus maenus*, parasitized by *Sacculina carcine*. He also noted tissue resembling adenosarcoma in the eye of the crab.

Several abnormalities observed in ticks, though confusing, could be considered a form of neoplasia or tumors. In a collection from Turkestan, Olenev and Rozhdestvenskaja (*19*) found 4 female ticks (*Hyaloma* sp., *Dermacentor niveus*) whose bodies were covered with numerous oval, whitish, well-defined, glossy-surfaced elevations called papules. One tick had about 1,000 papules, equally distributed on the dorsal and ventral surfaces, but confined to the more thinly chitinized parts of the body, thus being absent from the capitulum, scutum, and legs. Sections showed the papules were composed of chitin, but the cause and significance could not be explained.

Subsequently, Colas-Belcour (*3*) found lesions of the integument of 2 female ticks of the genus *Hyaloma.* The author noted these lesions were similar to those just previously discussed, and believed they probably resulted from an infection, but he could not demonstrate microorganisms in fixed material. He strongly emphasized the improbability that these obviously related lesions occurred by chance alone.

SUBPHYLUM UROCHORDATA

The only tumor or tumor-like growth reported among the ascidians is of parasitic origin. A gregarine, *Monocystis ascidiae*, spends the greatest part of its developmental period intracellularly in epithelial cells of the gut of a tunicate, *Ciona intestinalis.* Siedlecki (*24*) noted that infection of an intestinal cell initiated a hypertrophy, beginning with a slight enlargement of the cell, but with a distinct hypertrophy of the nucleus and condensation of the chromatin. As the gregarine grows, the hypertrophy of the cell is accentuated; the protoplasm lacks the density and homogeneity of normal cells and is studded with clear vacuoles, while the still enlarging nucleus is displaced into a corner of the cell. In subsequent stages the cell continues to enlarge, attaining 10–20 times its normal size, and the nucleus, after becoming smaller and very chromatic, disintegrates.

This response of the tunicate's intestinal cells is, of course, a clear case of cellular hypertrophy, as Scharrer and Lochhead (*23*) pointed out. However, Siedlecki reported that neighboring uninfected epithelial cells also proliferated and, in some cases, proliferation of surrounding connective tissue contributed to the formation of an abnormal growth which Siedlecki called a tumor similar to liver adenoma of rabbits. As Siedlecki noted, if one examines the advanced stage of the abnormal growth, the original cause, the gregarine, is missing and the growth thus appears to be a spontaneous neoplastic growth.

Siedlecki thought that the stimulus initiating the hypertrophy was chemical rather than mechanical. He theorized that, when the chemical agent was weak, only the parasitized cell would react, but when it was stronger, it would initiate proliferation of neighboring cells, resulting in tumor formation.

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Malformations and Lethal Growths in Planaria Treated With Carcinogens [1]

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SUMMARY

Seven and nine percent, respectively, of the adult planaria treated with benzo[*a*]pyrene and 3-methylcholanthrene developed lethal growths after forced regeneration. The offspring of the 3-methylcholanthrene-treated, regenerated worms developed lethal, papilliform tumors (30%) and malformations (32%). Twelve percent of the progeny of the benzo[*a*]pyrene-treated animals developed similar malformations. Implantation of paraffin-carcinogen pellets significantly increased the tumors in adult planaria. Evidence is presented that these abnormal, lethal growths may be derived largely from neoblasts, although some metaplasia of somatic cells is possible.—Nat Cancer Inst Monogr 31: 683–691, 1969.

PREVIOUS STUDIES have suggested that abnormal, lethal growths can be produced in planaria with carcinogens (*1*), the insecticides DDT and Sevin (*2*), and metabolic antagonists (*3*). The present investigation was intended primarily to study the effects of carcinogens on adult planaria, *Dugesia dorotocephala*. However, during the experiments reproduction occurred, and attention was also focused on the abnormalities of the progeny.

Spontaneous abnormalities do not occur in *D. dorotocephala* except for occasional black pigmented spikes. Spontaneous tumors have been reported in other species, however. The most recent report by C. S. Lange (*4*) described 54 spontaneous tumors in *D. etrusca* and *D. ilvana*. The development of these tumors was progressive and led to lysis of both tumor and host. Histologically, the cells and tissues did not appear to be abnormal, except for location. Lange suggests that, since neoblasts are the only cells in planaria which divide mitotically during regeneration, the abnormality

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is the result of faulty differentiation control rather than neoplasia. The simplicity of Turbellarian tissue, in which there is no vascular system (*5*), may be helpful in understanding the differences between vertebrate and invertebrate tumors.

METHODS

Carcinogens in medium.—To study the effects of carcinogens, experimental groups of 100 animals each were used. The worms, except for the control groups, were treated for 24-hour periods at weekly intervals by including 4 ml per liter of a saturated acetone solution of carcinogen, 3-methylcholanthrene (MCA) and benzo[*a*]pyrene (BP), per liter of BVT medium (*6*). Fifteen ml of a stock antibiotic solution was added to 5 gallons of BVT medium to minimize bacterial contamination. The stock antibiotic solution consisted of 1 million units of crystalline penicillin-G, 1 g (2.5 ml) streptomycin sulfate solution, and 100 ml distilled water. After 2 months, the worms, which had attained a size of 25–30 mm, were sectioned transversely into 3 segments. Ten days after sectioning, when regeneration was almost complete, they were subjected to an additional 24-hour treatment in carcinogen. Ten days later, many miniature planaria, accompanied by ruptured capsules and yolk materials, appeared in the medium.

Implants.—Since adult planaria in this study showed only a few lethal growths, a method was devised for the implantation of paraffin-carcinogen pellets. Twenty of the largest worms from a stock culture were selected. They averaged 5 mm wide and 25–30 mm long. Paraffin and benz[*a*]anthracene (BA) or MCA were implanted in 20 worms (10 worms for each carcinogen). The paraffin and carcinogen (4:1, w/w) were mixed together thoroughly. The heated mixture was allowed to ascend a heated capillary tube and was then drawn out into a thin thread on a piece of heavy foil. Pieces 1 × 1 mm were sectioned for implantation. The planaria were laid on a piece of aluminum foil on the surface of an ice cube and thereby immobilized. A small slit was made on the dorsal surface of the worm with irridectomy scissors, and the paraffin-carcinogen pellet inserted into it. Worms were then transferred back to BVT medium. Complete healing took place very rapidly. After 2 months, the heads and tails were sectioned off and the center segments of the worms retained.

RESULTS

Weekly Treatment With Carcinogens in Medium

Nine percent of the MCA and 7% of the BP-treated adult animals developed tumors. Figure 1 shows a typical tumor from an adult MCA-treated animal. Grossly, the tumor was about one-third of the length of the worm. It arose from two stalks on the dorsal surface of the tail. Histologically, there were considerable disorganization of tissue elements and vacuolization of cells. Underlying an area of extensive epithelial hyperplasia and hypertrophied muscle was a large area composed entirely of cells containing pigment granules. The central tumor area contained gland cells and cells with pyknotic nuclei and large globules of pigment. In addition, nests of basophilic neoblasts were seen, leading to the belief that at least part of the growth had arisen from neoblasts. The digestive tract of planaria normally consists of a one-cell layer of gastrodermal cells. This had been replaced entirely by cells containing many pigment granules.

Offspring

Normally, planaria remain encapsulated from 2–3 weeks (*5*). The offspring in the present study were unusual in this respect, in that they emerged from the capsules much earlier. This phenomenon may be attributed to temperature or to some aspect of the culture medium, since reproduction occurred in untreated as well as treated animals (table 1). Malformations were most frequent in the offspring (40) of the MCA-regenerated group where 13 malformations and 12 papilliform tumors were observed (*see* table 1 and fig. 2). Fewer tumors and malformations were found in the BP-regenerated group, and acetone-treated and untreated animals had some malformations. Malformations related to the development of head and eyespots indicate a very early influence of the carcinogen on the differentiation of the neoblasts.

Attention was focused on 12 offspring of the MCA-treated adults who very early showed heavily pigmented knobs on the dorsal surface (*see* fig. 3). These animals rapidly surpassed the normal progeny in size, and pigment cells were lost in the head and tail regions, suggesting a migration

TABLE 1.—Lethal growths and developmental anomalies in the offspring of planaria treated with carcinogens

Treatment of parents	Number of offspring	Growths in offspring	Malformations in offspring
MCA, regenerated	40	12 papilliform tumors	6 eyespots poorly developed 4 enlarged heads and eyes 3 fused eyespots
BP, regenerated	75	2 nodular tumors	3 enlarged heads and eyes 2 small heads and eyes 2 fused eyespots
Acetone, regenerated	65	1 nodular tumor	Variation in pigmentation 2 enlarged heads and eyes
Untreated controls, whole	77	None	1 small head and eyes 1 fused eyespots
Untreated controls, regenerated	None		
MCA-treated, whole	None		

of pigment cells into the stalk of the tumor (fig. 2). Histologically, 14 days after capsule eruption, the knob consisted of pigment cells, epithelium, and a central core of neoblasts. The animals were thickened centrally and the digestive gastrodermal cells showed hyperplasia (fig. 4). Among the latter were some large, very basophilic nuclei surrounded by a thin area of deep-blue cytoplasm (hematoxylin and eosin stain). Tabs of tissue extending into the digestive tract (fig. 5) contained many of these basophilic cells, which were not seen in this area in normal controls. After 2 weeks, the progeny having these papilliform growths and gastrodermal abnormalities began to regurgitate beef liver into the medium. On the 20th and 21st days large, bulbous tumors (fig. 2) had developed on the dorsal stalks and the 10 remaining animals, which had not been sectioned histologically, died.

Implants

Tumor incidence in adult animals was increased markedly by implantation of paraffin-carcinogen pellets. Five of the ten MCA-implanted animals developed heavily pigmented growths, and four of the BA-implanted animals had nonpigmented nodular growths. These growths were not sectioned histologically and all were lethal to the planaria. One BA implant underwent fission at 1½ months. Rather than develop a normal tail, the head section of the animal formed another head at the opposite end (fig. 6), indicating a reversal of the normal head-tail polarity. Some of the MCA-implanted animals developed heavily pigmented growths with small, secondary, bulbous, nonpigmented growths on their surfaces (fig. 7). One BA-implanted animal showed multiple nodular growths and a large, nodular growth bordered by two normal secondary heads (fig. 8).

DISCUSSION

The presence of nests of basophilic neoblasts in the central tumor area of MCA-treated adult worms, as well as the number of neoblasts in the stalks of the papilliform tumors of offspring of these animals, strongly suggests that these abnormal growths arise from totipotent neoblasts. This agrees with the observation that vertebrate carcinogens generally stimulate regenerative cells in the invertebrates (*7*). The origin of the pigment in the gastrodermal cells of MCA-treated adult animals is in question. This finding may represent a metaplasia of gastrodermal cells or, possibly, a condition similar to melanosis coli (*8*) in vertebrates, in which there is a brown or black discoloration of the intestinal mucosa due to stasis of intestinal contents. This stasis is believed to result in absorption of protein disintegration products, which are converted into pigment by a tyrosinease-like enzyme. It was noted that the adult MCA-treated worms failed to extend the pharynx during later feedings, and the second explanation seems more likely. Harshbarger and Taylor (*9*) report that a similar finding in the gut of insects is sometimes erroneously described as a tumor.

It seems probable that pigment cells, as well as neoblasts, migrate in the planaria, since, while the stalk of the bulbous tumor was growing in the offspring, the tail and head regions were undergoing depigmentation. Evidence of migration of pigment cells to the gut is reported in a paper on the induced loss of pigment by planaria treated with fungichromin (*10*). It is interesting to find heavily pigmented tumors in both offspring and adult MCA-treated animals. 3-Methylcholanthrene has been found to produce melanotic tumors in vertebrates (*11*). The origin of the deeply basophilic cells in the gastrodermis of the progeny is not known. The cell seems to have no counterpart in the normal control animal, unless it should be the filamentous pigment cells that normally lie in the epidermis. They do bear some resemblance to cells at the base of stalk of the tumor in the offspring.

The development of the small, secondary, nonpigmented growths on the primary, heavily pigmented growths in MCA-implanted animals seemed to represent abortive attempts to form secondary heads and is further evidence of the existence of active, totipotent neoblasts in the area. One animal with a BA implant developed two secondary heads bordering a large nodular growth (fig. 8), suggesting that the neoblasts might be involved simultaneously in both normal and abnormal growth patterns.

The action of paraffin in enhancing the activity of MCA and BA is considerable. Whether this is due to slow release of carcinogen, trauma, or the promoting activity of the paraffin itself is not known.

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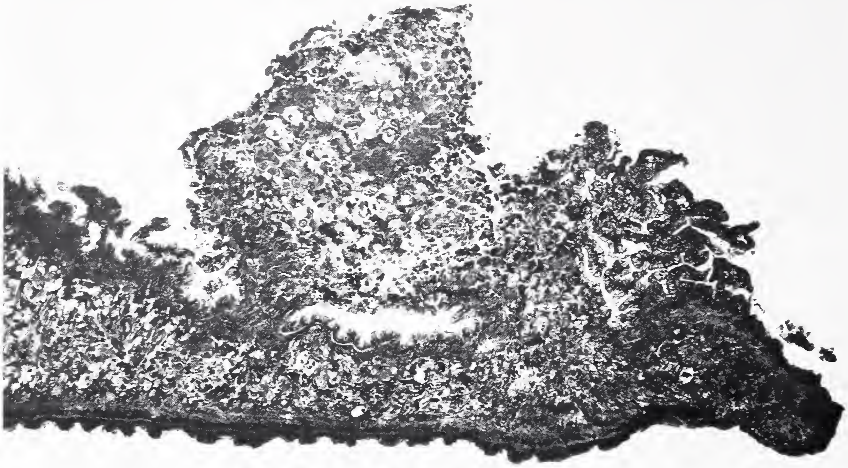

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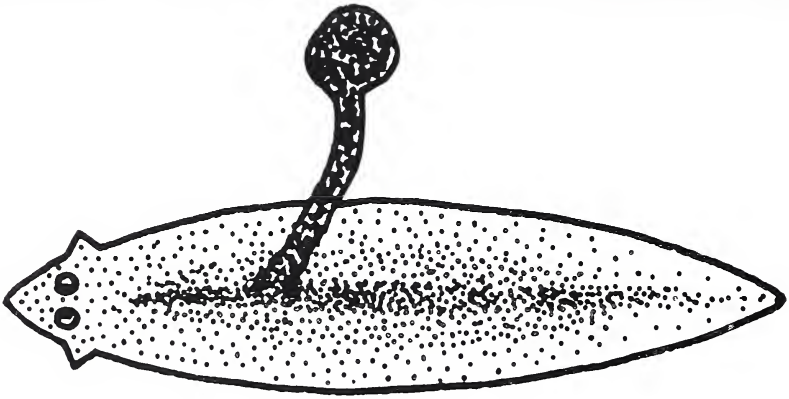

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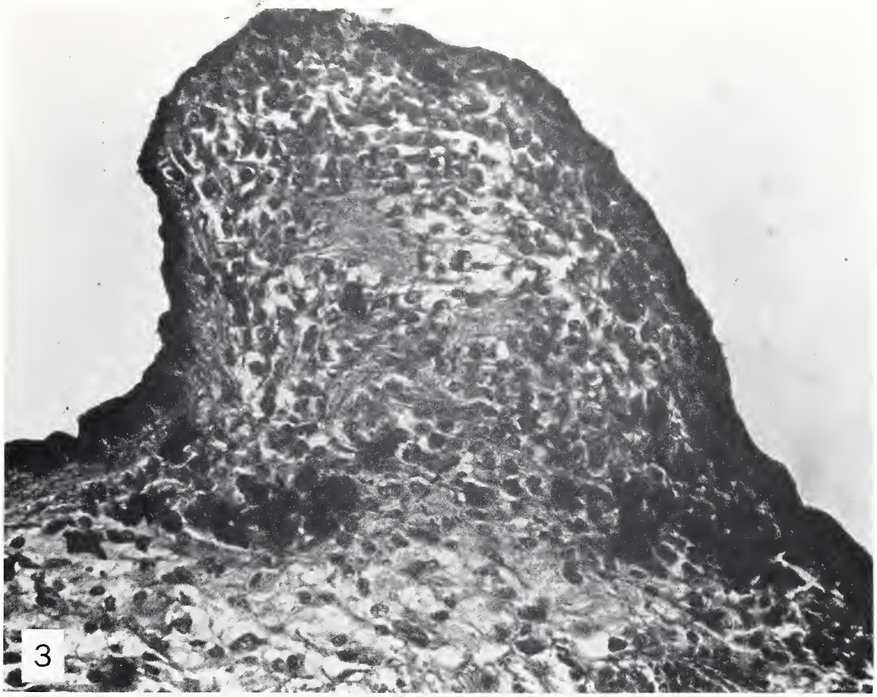
Figure 1.—Sagittal section of adult worm treated with 3-methylcholanthrene. Extensive areas of epithelial hyperplasia and pigmentation. Center tumor area contains nests of neoblasts.

Figure 2.—Papilliform tumor on offspring of 3-methylcholanthrene-treated adult animal. At 21 days, extensive depigmentation of remainder of animal had occurred.



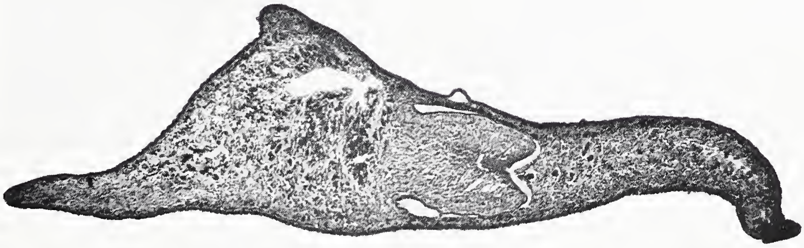
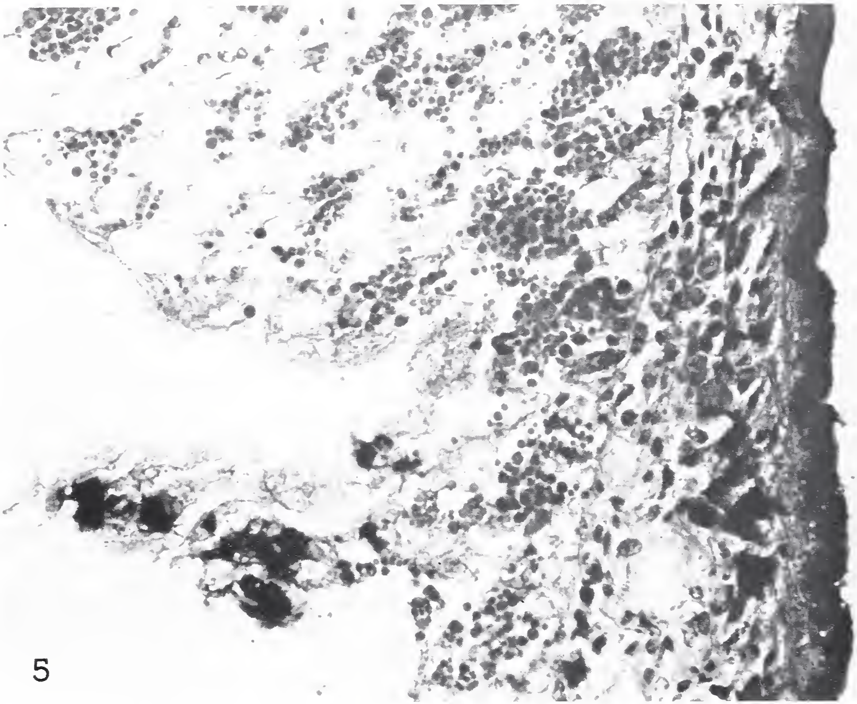


Figure 3.—Stalk of tumor in the offspring at 14 days contained epithelial cells, pigment cells, and a central core of neoblasts.

Figure 4.—Sagittal section of offspring of 3-methylcholanthrene-treated adult. *Note* extensive gastrodermal hyperplasia below lumen of digestive tract.



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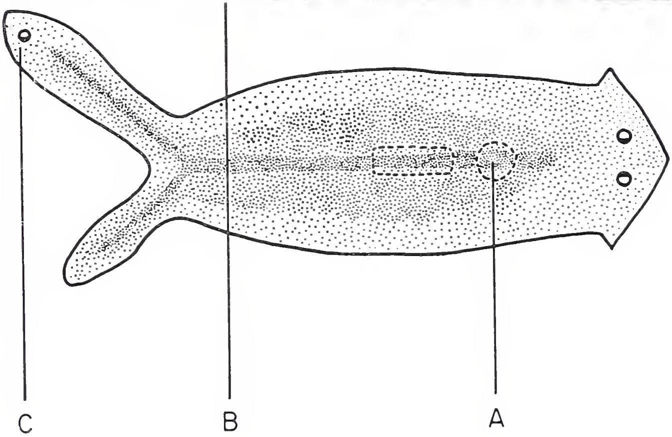

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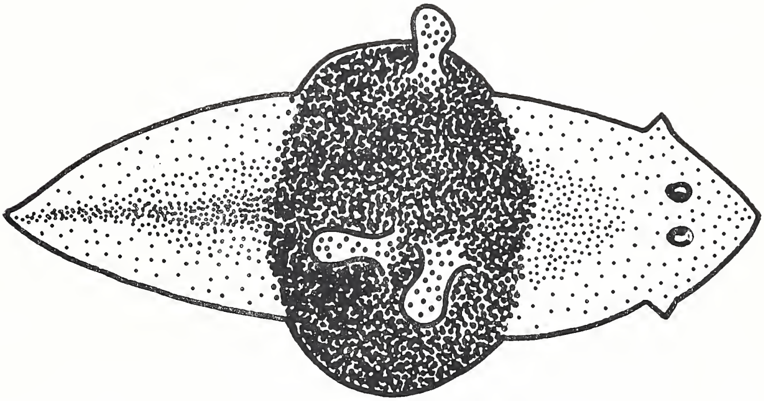
FIGURE 5.—Tab of tissue in the gastrodermis of the offspring of 3-methylcholanthrene-treated adult showing large, basophilic nuclei.

FIGURE 6.—Benz[*a*]anthracene implant: A) implantation site; B) region of fission at 1½ months; C) head development in normal tail development region.



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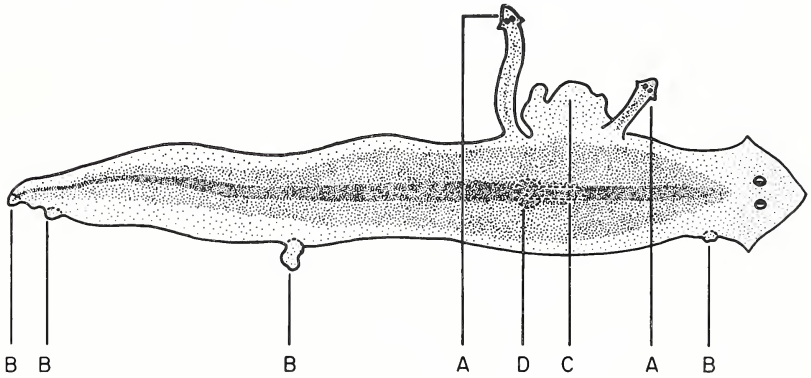

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FIGURE 7.—3-Methylcholanthrene implant. Heavily pigmented growth with several nonpigmented, secondary growths.

FIGURE 8.—Benz[*a*]anthracene implant: A) secondary heads; B) and C) multiple nodular, nonpigmented growths; D) approximate implantation site.

Histopathological Effects of Ionizing Radiation on the Planarian, *Dugesia tigrina* [1]

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SUMMARY

This study describes the histopathological effects of various doses of γ radiation in *Dugesia tigrina*. Alterations in gross appearance are also described. Ulcers occurred in all groups of animals beyond the 1000 r dose group, and a "liquefied head" syndrome developed in all but the control and 1000 r group. Histopathological changes observed in the lower level dose groups (1000 r, 5000 r, and 10,000 r) were generally similar, differing only in time of appearance. These changes consisted of decreasing numbers of neoblasts with a corresponding thinning of the parenchyma, followed by pathological changes in the intestinal epithelium, epidermis, and glandular tissue. At the higher levels (50,000 r and 75,000 r) death was rapid and relatively few pathological changes were noted.—Nat Cancer Inst Monogr 31: 693–707, 1969.

STUDIES OF THE EFFECT of irradiation on planarian regeneration have been conducted for more than half a century. Bardeen and Baetjer (*1*) found that regeneration was completely inhibited by cumulative doses of X rays (doses not given). Death, occurring in 20–30 days, was ascribed to a degenerative process which began in the anterior region and spread posteriorly, but no histological alterations were described. Schaper (*2*) and Weigand (*3*) obtained similar results using radium. Weigand reported that mitosis was inhibited and disintegration of chromosomes followed. He also described "giant cells" which Dubois (*4*) considered analogous to giant neoblasts filled with fragmented chromosomes which she found in her work. Curtis and Hickman (*5*) and Curtis (*6*, *7*), using X rays and radium, found that planaria continued to live for some time after irradiation, but did not regenerate. They also agreed that "embryonic formative cells" (neoblasts) were most sensitive to X rays. Wolff and Dubois (*8*) and Dubois (*4*) in a classic study elaborated on the functions and abilities of the neoblasts. Dubois found that with whole-body irradiation, after amputation of the head, regeneration of the eyes occurred, but a normal head would not be regenerated. She established that the threshold dose inhibiting regeneration was between 2500 r and 5000 r (with the planaria being slightly more resistant in spring and summer) and that no regeneration occurred if the dose was greater than 8000 r.

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Dubois also noted that mitoses occurring at the moment of irradiation proceeded normally, but mitoses post irradiation were abnormal and fragmentation of the chromosomes occurred, followed by the death of the cell. Chandebois (*9*) found the minimal dose that would destroy all cells in mitosis is about 5000 r. Dubois determined by localized irradiation that neoblasts are totipotent and are able to migrate (speed varying directly with temperature) and to form the regenerative blastema. Wolff and Lender (*10*) also showed that whole-body X irradiation with doses of 5000 r prevents regeneration and the animals die after 3–7 weeks. Hashlauer (*11*), however, found that, in subjecting planaria to lethal doses of ultraviolet light, regeneration could not be completely inhibited, and small radiation doses distributed over the entire course of regeneration significantly stimulated the regenerative growth. Le Moigne (*12*) discovered that X irradiation destroys the neoblasts during the embryonic development of the planaria and suppresses regenerative capacity without inhibiting the differentiation of the rudiments already determined at the time of irradiation.

Although radiation has been used extensively in planaria research, it has not been reported to cause tumors. This may be because changes in the control mechanisms of the cell require long periods (years in mammals) before they become manifest in the altered genetic structure that characterizes neoplasia (*13*).

Stephan (*14*) reported spontaneous tumors which appeared as massive infiltrations of glandular cells in the parenchyma, and Lange (*15*) described tumors which consisted of papillary and cryptlike structures covered and lined by a mucus-producing epithelium and a broken perforated membrane, and which contained large pockets of normal neoblasts. Lange suggested these may have been the result of differentiation error rather than an actual neoplasia.

Seilern-Aspang (*16, 17*) discussed tumors caused by accidents during regeneration in planaria. Several tumors or tumor-like outgrowths caused by cauterization or carcinogens have been reported. Goldsmith (*18*) described supernumerary headlike growths produced in *Planaria maculata* with the use of ethyl alcohol, aniline oil, and coal tar. Foster (*19*), using *Dugesia dorotocephala* treated with carcinogens, observed nodular growths which increased in size, became necrotic, and eventually caused the death of the animal. She suggested that these growths be considered neoplastic. Stephan and Schilt (*20*) also described supernumerary formations induced by cauterization. These tumor-like growths were characterized by acidophilic and siderophilic hypersecretion. Some were innervated.

MATERIALS AND METHODS

Planaria (*Dugesia tigrina*), collected from ponds in the arboretum on the University of Washington campus, were divided into 7 groups of 60 animals each. They were maintained throughout the experiment in a semidark room, at a temperature of 62° ± 2°F, in 100 × 50 mm crystallizing dishes in which the water was changed every other day. Each group received a different dose of γ radiation from a Mark II food irradiator which utilizes a cobalt-60 double plaque of approximately 34,000 curies of cobalt-60. Doses given were (in rads): 0; 1000; 5000; 10,000; 25,000; 50,000; and 75,000. All specimens were fixed in Zenker's at 24, 48, 96, and 150 hours, 17 days, and 4, 6, 8, and 10 weeks.

RESULTS

Gross

The 75-kilorad (kr) group showed dramatic effects immediately upon removal from the irradiator. Many of these animals twisted uncontrollably and fell from the sides of the container. Within 2 hours, large concentrations of mucus had appeared in the dish, and none of the animals showed any avoidance response to light. At 12 hours, one animal had died with a large ulcer covering half the midportion of its body. By 24 hours, another had died, several had ulcers in various areas, and a few had clear "polyp-like" outgrowths on their bodies. By 48 hours, many of the remaining animals had distorted body shapes, with some areas, usually prepharyngeal, greatly enlarged due to the accumulation of fluid. By 72 hours, few remaining planaria appeared normal. Anomalies were frequently observed in the head area. Some animals had reduced heads, with ulcers about the eyes, and several had large ulcers in the prepharyngeal areas. By 96 hours, 20% of this group were dead or disintegrating, and all had gross abnormalities, especially in the anterior regions. These abnormalities ranged from fragmenting eyes to loss of eyes and liquefaction of the head. The occurrence of abnormalities continued to increase in the remaining specimens, and by 144 hours all were dead.

Except for the control and 1000 r groups, a fairly definite pattern of gross changes evolved which is referred to as the "liquefied head" syndrome. Initially, ulcers appeared on the body, usually in the middorsal or posterior-dorsal regions (fig. 1). The time of appearance varied according to dose (*e.g.*, in the 50 kr group the ulcerations appeared within 50 hours, but at 5 kr not until the 20th day). A period followed in which ulcers were found in the anterior regions and around the eye cups. The ulcers were outlined by darkly pigmented peripheries consisting of necrotic tissue (*see* fig. 2). The eyes then began to fragment and eventually disappeared.

Immediately after this (or in some cases while the eyes were fragmenting), the head began to lose its normal shape and eventually became a rounded mass of liquified tissue. This necrotic area was then shed, leaving a pigmented scar ("necrotic collar," according to some French authors). The time of appearance of this liquefied head varied from 144 hours after 50 kr to 24 days after 5 kr. One case of apparently normal regeneration of the anterior region occurred in the 10 kr group and two cases of abnormal regeneration, one of which will be described, occurred in the 5 kr group.

Microscopic—Control

The animals in the control group appeared histologically normal throughout the experiment, although some had atrophic changes undoubtedly associated with starvation. Mitotic figures (usually metaphase plates) were observed in parenchymal cells (*see* fig. 3) in control animals but not in the irradiated groups after 24 hours.

The parenchyma was consistently occupied by many neoblasts of varying sizes, a characteristic that always distinguished normal from experimental animals, which contained no small neoblasts. At 1 month the parenchyma began to appear slightly less cellular, and a gradual decrease in neoblast numbers was generally observed until termination of the experiment. Except for parenchymal rhabdite-producing cells, which were slightly reduced in size by the 17th day, there were few changes in any tissue until 1 month. Eosinophilous and cyanophilous glands were observed in the parenchyma and lateral marginal areas throughout the experiment, although there appeared to be a decrease in gland cell numbers, also beginning by the 17th day. The intestinal epithelium retained its typical acidophilic staining reaction, and the epithelial cells did not change noticeably (excepting a decrease in size) until 10 weeks, when bands of light basophilic granular-appearing material and neoblasts began to surround the intestinal loops.

Irradiated

1000 rad

A few neoblasts became necrotic by 24 hours, most of the dead cells having apparently undergone karyorrhexis and developed irregular cell membranes. Except for a general decrease in neoblast concentration in the parenchyma, few pathological changes were observed in this group until the 17th day, when the intestinal epithelium in one animal showed increasing eosinophilia and granularity.

By 1 month the intestinal branches and internal parenchymal tissues were intensely eosinophilic and the intestinal branches were surrounded by basophilic granular material (fig. 4). Gland cells, when clearly distinguishable (*i.e.*, when it was possible to observe their necks protruding through the epidermis), were almost black. The epidermal cells presented a somewhat confusing picture. Some appeared to have undergone hypertrophy while others appeared small, in comparison to normal cells. Visible neoblasts were generally confined to the area immediately adjacent to the nerve cords (fig. 5), but some were concentrated in the ventral margins. Rhabdite-producing cells were infrequently observed and were reduced in size. By the 6th week, regeneration of injured tissues was nearly completed, and by the 8th week, all tissues were normal and the parenchyma had regained its cellular appearance.

5000 rad

Changes were few, again with the exception of a decrease in neoblast numbers, until 150 hours. At that time, the dorsal-ventral parenchymal muscles had begun to lose their regular architecture, becoming shorter and wavy. Rhabdite-producing cells were absent or greatly reduced in all but one animal. The intestinal epithelium in one animal appeared to have undergone metaplasia, and the cell boundaries were no longer distinguishable. In the other two, the gut epithelium had become granular.

By the 17th day, the parenchyma had become markedly diffuse and, due to the lack of neoblasts, was virtually acellular. The internal parenchymal area contained large amounts of an intensely staining acidophilic material which was often surrounded by a dark, thin basophilic border. In most cases the intestinal epithelium was somewhat granular with indistinguishable cell boundaries.

At 1 month, 2 animals developed infolded crypts, lined by epidermis containing many rhabdites (fig. 6). Rhabdites were absent in all other epidermal areas. Both of these animals had an apparent hyperplasia of dorsal muscle cells. Epidermal cells in all animals of this group and at this time were abnormal in shape and size: Some cells were greatly enlarged and contained large nuclei, whereas others were smaller than normal. The intestinal epithelial cells were poorly defined, appearing granular and possessing faintly staining nuclei. The parenchyma appeared as a homogeneous mixture of intestinal epithelial fragments and eosinophilic spherules associated with the "club" cells of the gut epithelium (fig. 7).

The only survivor in this group at the end of 8 weeks had undergone abnormal regeneration. Only one nerve cord was present in the anterior region just posterior to the brain. Muscles and intestinal epithelium were disorganized and in abnormal locations (*e.g.*, one intestinal branch was on the extreme middorsal surface). The center of the animal contained large clusters of normal neoblasts and an acidophilic debris-like mass surrounded in part by elongated neoblasts. The second nerve cord became evident in the prepharyngeal area. There, the dorsal epidermis consisted of squamous-like cells rather than the normal columnar epithelium. The large concentration of middorsal neoblasts was still evident. The pharynx in this animal was absent, although a reduced pharyngeal cavity was present, lined by an acidophilic, cuboidal epithelium. This cavity was filled with debris in the postpharyngeal area. The parenchyma in the postpharyngeal area was relatively normal, containing neoblasts and rhabdite-producing cells.

10,000 rad

At 24 hours, a lack of neoblasts in the parenchyma was conspicuous, but normal numbers of neoblasts appeared around the periphery of the parenchyma. Through 150 hours, the pathological changes paralleled those of the 5000 r group.

By 17 days, the few remaining neoblasts were confined to the areas adjacent to the nerve cords. The condition of the intestinal epithelium varied, being normal, paler staining, reduced (metaplastic), and sloughing in different animals.

In an intestinal branch of one animal, the epithelium was absent or greatly reduced and all that remained was a mesothelium-like lining whose nuclei bulged into the lumen. It appears in all these animals that the branches of the gut furthest away from the middorsal-ventral plane (those toward the lateral surfaces) showed more severe pathological changes (*e.g.*, sloughing, metaplasia) than those located toward the center of the animal. This was also true in the groups given the higher doses. Dorsal-ventral parenchymal muscles were less thick and the fibrils sometimes separated. No rhabdite-producing cells were observed in the parenchyma.

By 1 month, the nerve cords appeared granular and in some specimens were virtually indistinguishable from the surrounding parenchyma. There was no neoblast concentration around the nerve cords, although a few neoblasts were scattered throughout the margins. The internal tissues and parenchyma appeared as thick, intensely eosinophilic homogeneous masses surrounded by basophilic granular borders. In those animals where epidermal-muscle borders were distinguishable, the borders were separated by a granular basophilic substance that ranged from blue to black (fig. 8). One animal had an epithelium-lined invagination or crypt on the dorsal surface. Gut epithelium was unidentifiable in all but two animals, and in another there appeared to be an encapsulation attempt around the necrotic intestinal epithelium. Elongated cells (neoblasts), 1–2 cell-layers thick, surrounded this particular branch.

25,000 rad

Many granular, basophilic strands appeared in the cephalad parts by 24 hours in two animals. They extended posteriorly to the prepharyngeal area. An apparent hyperplastic outgrowth was observed on the dorsal lateral surface of one animal at this time. It consisted of a large, homogeneous, eosinophilic mass extending from the epidermis to the midbody region. It apparently displaced rhabdite-producing cells, since they appeared on either side but not in the growth below it. The matrix of the hyperplastic growth was markedly cellular, containing neoblasts and some cells too small to be neoblasts.

By 96 hours, all eosinophilic glands except those along the margins were absent. By 150 hours, large amounts of granular, basophilic strands appeared throughout the body in all animals. Viable neoblasts were confined to areas immediately surrounding the nerve cords and the brain.

The intestinal epithelium was necrotic and in various stages of being sloughed in all but one large specimen. No rhabdite-producing cells were observed in any experimental animals in this group.

50,000 rad

Several animals in this and the 75,000 r group had midbody dorsal-ventral constrictions, beginning in the postpharyngeal area and proceeding posteriorly. Histologically, the constricted area in the middle of the animal was filled with viable-appearing neoblasts, while all neoblasts outside this central area were necrotic by 24 hours. One animal (at its anterior end) appeared to be in the initial stage of the infolding observed in other animals in lower dose groups after longer intervals. There were many small rbabdites, appearing fragmented, in the constricted surface. One rhabdite-producing cell in this area was greatly enlarged and filled with many small rhabdites. Necrotic neoblasts were present and appeared to have undergone karyorrhexis, with nuclear membranes wrinkled and little chromatin material visible.

Most viable neoblasts were at the anterior ends of the worms, surrounding the nerve cords. The parenchyma contained unusual eosinophilic cells. These were not gland cells, nor did they appear to be neoblasts, because of their large size. Each contained a large vacuole, and one was in mitotic prophase (M. Ahmed, personal communication).

Large concentrations of basophilic, granular strands began to appear at the anterior end by 24 hours. This material increased through 48 hours, and by 96 hours was found throughout the body. Viable nests of neoblasts were observed in pharyngeal areas; many rhabdite-producing cells were fragmented and the rhabdites had been released into the parenchyma. A decrease in eosinophilous glands was conspicuous throughout the body.

By 150 hours, these animals were so deteriorated that further histological study was difficult. The parenchyma was virtually acellular (fig. 9), although there were some viable-appearing neoblasts surrounding the nerve cords. Most animals possessed no gland cells. The intestinal epithelium was necrotic and in the process of being sloughed from the basement membrane.

75,000 rad

Histological changes in this group were identical to those observed in the 50,000 r group through 96 hours. By 96 hours, the parenchyma in several animals appeared vacuolated with large clear areas. As rhabdite-producing cells were greatly reduced in size, a clear halo remained around each of these cells.

DISCUSSION

Although many studies concerning regeneration and the role of the neoblast in forming the regenerative blastema have been carried out by Dubois and Wolff (*4*, *8*) and reviewed by Brøndsted (*21*) and Kiortsis and Trampusch (*22*), there is, even today, considerable controversy over this issue. Dubois (*4*) described the blastema of regeneration as being filled with neoblasts that differentiate into epidermal cells, muscles, cerebral cells, eye cells, etc. Wolff and Dubois (*8*), using X rays on the prepharyngeal region of a decapitated planarian, demonstrated the ability of the neoblasts to migrate. Four weeks after the amputation, a new blastema was formed and the animal regenerated normally. Animals that received whole-body irradiation all died without regeneration. Wolff and Dubois explained that, in the locally irradiated animals, the colonization of the irradiated portion was assured by the neoblasts which migrated from the nonirradiated region and that the 4-week delay was necessary for the cells to "succeed in their purpose."

Lender (*23*) also described the formation of the blastema as the accumulation of neoblasts: Under the influence of a stimulus provided by the injured tissues, these cells multiply by mitosis and migrate toward the wound. Then the neoblasts differentiate in order to build the regenerated tissues.

Chandebois (*24*) disclaimed the neoblast concept of regeneration, offering instead a slightly more complex idea of a "cell transformation system." Briefly, he stated that planarians possess permanent totipotent cells in their parenchyma, but that these are fixed elements, apparently fused in a syncytium, which can be produced by dedifferentiation of differentiated cells. The free cells initially accumulate near the wound, undergo amitosis, and are finally destroyed by histolysis. They act in regeneration by providing the syncytium with RNA, which is necessary for specific synthesis in the regenerate.

It was not our purpose to conduct another study on regeneration, but our results provide some interesting points. Apparently normal regeneration was achieved by planaria receiving whole-body radiation doses in excess of those used by Dubois (*4*) and Wolff and Lender (*10*). This may serve to show that studies utilizing radiation on planaria (or, in fact, any invertebrate) should be repeated several times. Because they are poikilothermic and asexual, variable results may be expected at different temperatures and during different periods of their reproductive cycle. The appearance of the unidentified basophilic materials in all irradiated animals (and a few controls) is intriguing, and histochemical techniques may reveal their nature. There are four possible origins of this substance: 1) from necrotic parenchyma, 2) from proliferating glandular cells, 3) from hypersecretory activity of already existing glandular cells, or 4) from nucleic acids released from cytolyzed neoblast or parenchymal cells.

This preliminary study has laid the groundwork for further experiments. The "liquefied head" syndrome was observed consistently in all animals irradiated with the lower doses. Using whole-body doses of 5000 and 10,000 r to achieve this gross effect should aid in further studies concerned with regeneration, possibly answering the questions of whether normal regeneration can occur if all neoblasts are destroyed and whether all neoblasts are, in fact, destroyed. Analysis of the basophilic substance may also shed light on this problem. Finally, because the histopathological changes were variable in each group, establishment of a clone from a single animal and use of only two or three levels of radiation (representing acute and chronic levels) may aid in producing consistent results.

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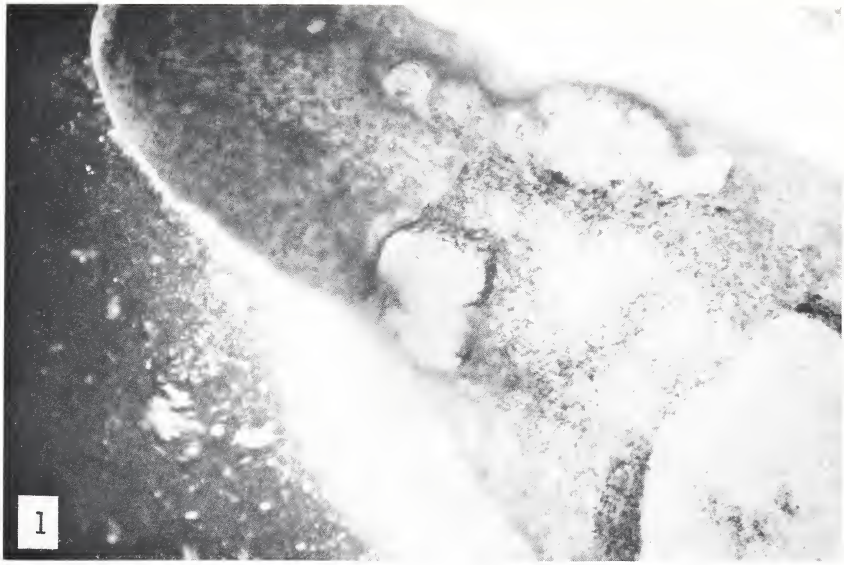

Figure 1.—Ulcer in posterior. × 2.5

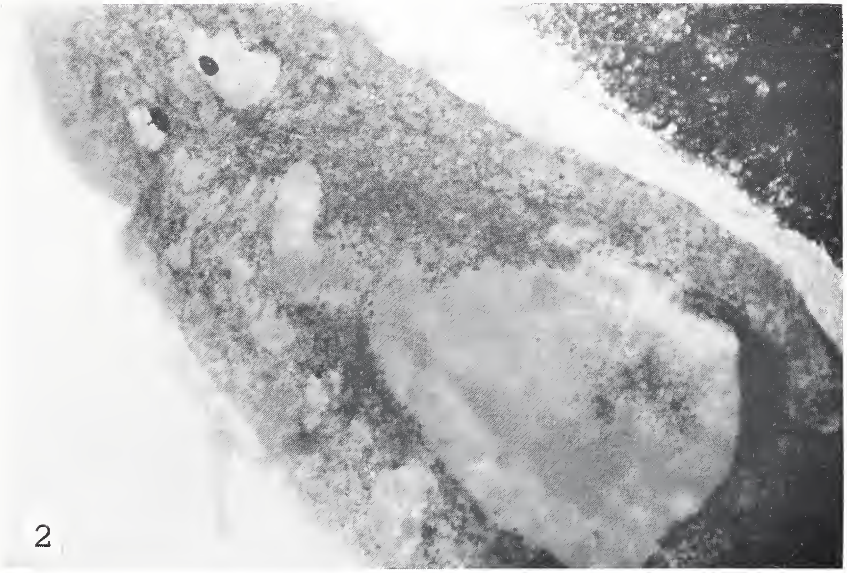

Figure 2.—Ulcer in middorsal region. *Note* dark periphery consisting of necrotic tissue. × 2.5

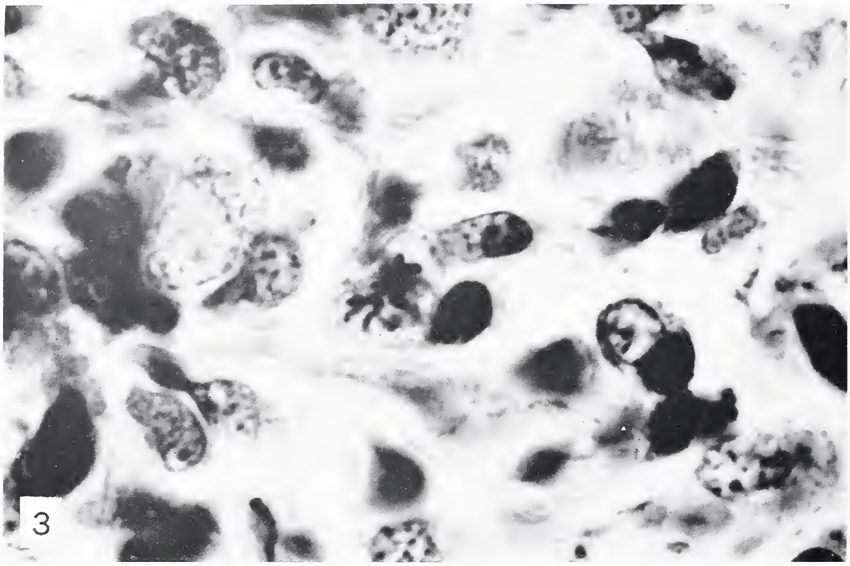

FIGURE 3.—Mitotic figure (metaphase plate) apparently in parenchymal cell. Unirradiated animal. × 1740

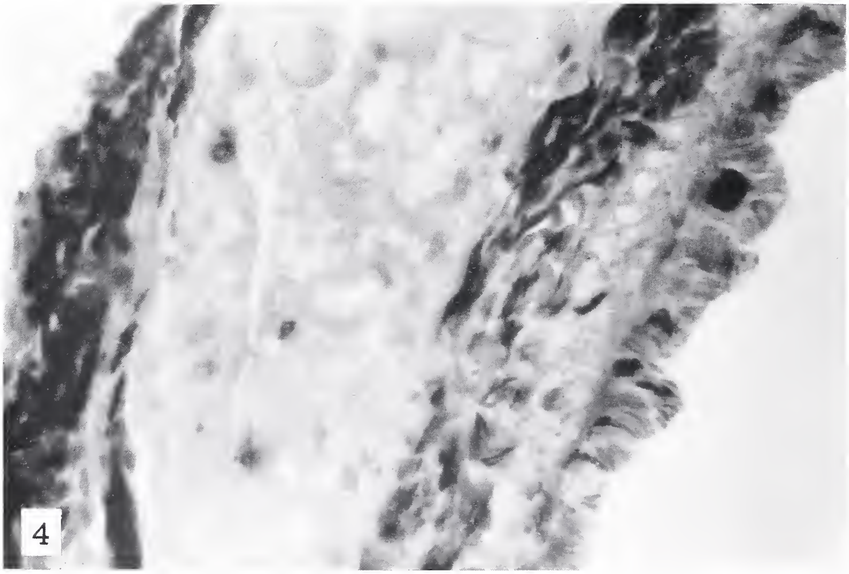

FIGURE 4.—Necrotic intestinal loop surrounded by unidentified basophilic, granular substance. 1000 rad. × 6696

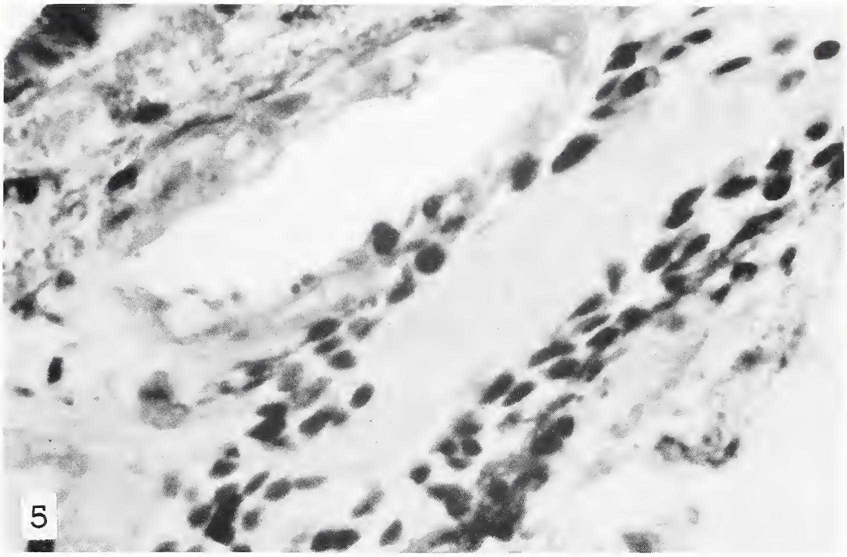

FIGURE 5.—Nerve cord surrounded by viable-appearing neoblasts. 1000 rad. × 544

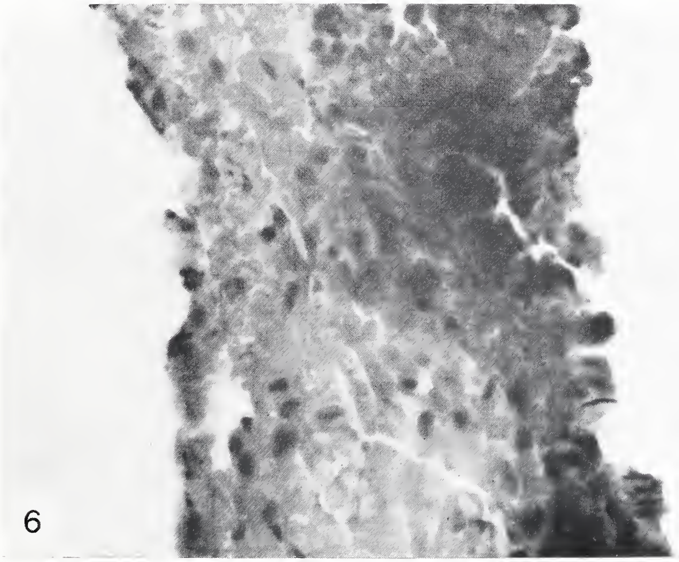

FIGURE 6.—Infolded crypt lined by epidermis containing many rhabdites. 5000 rad. × 696

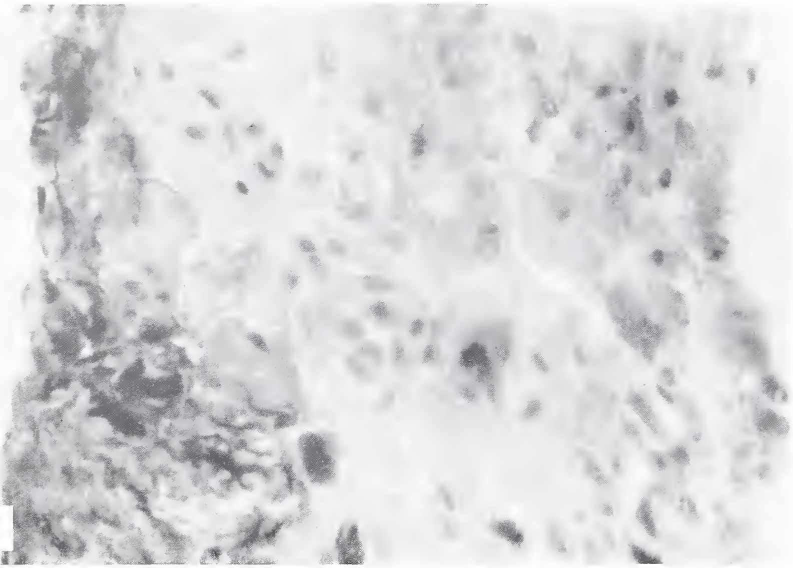

FIGURE 7.—Parenchyma after 1 month (55 rad). *Note* intestinal epithelial fragments and eosinophilic "spherules," which are associated with club cells of gut epithelium, scattered throughout. × 696

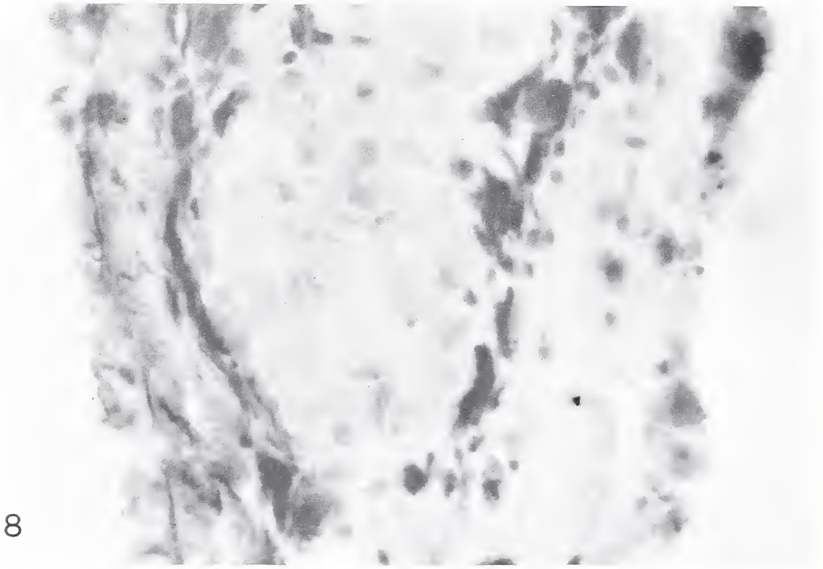

FIGURE 8.—Unidentified basophilic material observed throughout parenchyma and at epidermal-muscle borders. 10,000 rad. × 696

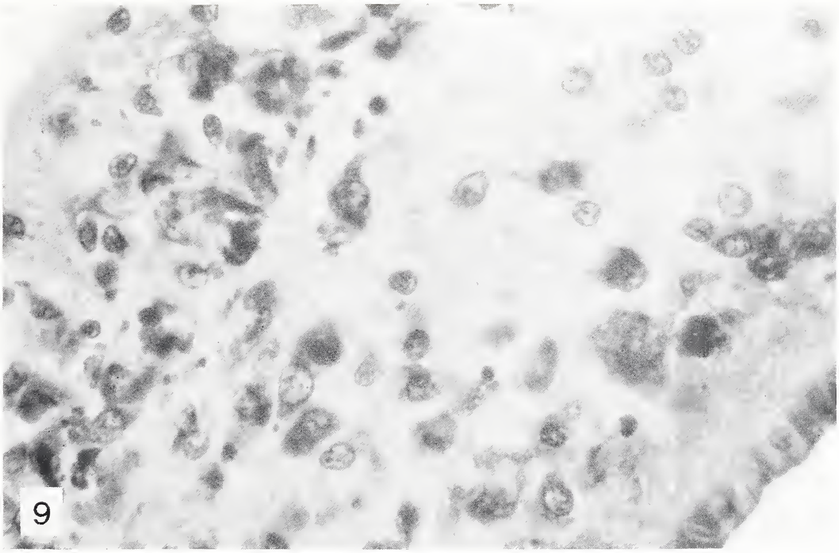

FIGURE 9.—*Note* parenchyma appearing virtually acellular. 50,000 rad. 150 hours. × 696

Anomalies of Growth and Form in Hydra: Polarity, Gradients, and a Neoplasia Analog[1]

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SUMMARY

Growth anomalies and a neoplasia analog in hydra are discussed in relation to polarity, a reversal of polarity in normal hydra, an atypical polarity in a mutant hydra, and the possible involvement of metabolic gradients. The presence of a beginning bud on an animal that is transected just distal to that bud leads to the formation of a lateral hydranth animal. In this lateral hydranth animal normal regeneration is inhibited, the polarity of the distal part of the main body column is reversed, and a new lateral center of polarity develops. A mutant of hydra is described. It does not reproduce by budding; its size is ten times that of normal hydra, and often it takes on multipolar shapes, especially during regeneration. Heterocytes were derived from grafts made between normal and mutant hydra. In normal-looking heterocytes, mutant somatic cells appear to proliferate, segregate, come off as a bud, and express their phenotype in those buds. Eventually the normal-looking heterocyte changes its morphological and reproductive characteristics and becomes gradually transformed into an animal of mutant phenotype. The possible relations to a primitive neoplasia of these interactions between normal and mutant cells are discussed. As a means for investigating the view that metabolic gradients are related (directly or indirectly) to the polarity of hydra, the Lowry microbiochemical analytical procedures were used. The results show that each of the two nicotinamide adenine dinucleotide phosphate-reducing enzymes, glucose-6-phosphate dehydrogenase and isocitrate dehydrogenase, has specific patterns of distribution along the body column.—Nat Cancer Inst Monogr 31: 709–737, 1969.

FEW ANOMALIES of growth have been described in coelenterates other than hydra, with one notable exception: the descriptions of a possible neoplasia in a coral (*1*). Perhaps a prime reason for the interest in growth

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anomalies in hydra is that among the coelenteraes, the hydras are still the organisms most adaptable to experimental research. The animal appears to be relatively simple; it is diploblastic and contains around 7–9 major cell types. Furthermore, the animal can be grown in clones through asexual reproduction by buding, in mass culture (*2*, *3*), and under relatively controllable conditions. Yet a major difficulty in investigating abnormal growth and form in hydra is that the animal has a remarkable facility for regulating anomalies resulting from external chemical or physical influences—and such anomalies soon disappear.

Most discussions of the regulative abilities of hydra, and of growth and form in general, usually revolve around the topic of polarity—a term which basically means the symmetry or organization of an animal (*4*). Hydra are said to have an apical-basal type of polarity; that is, the head is always at the distal end and the base is always at the proximal end. Furthermore, the polarity of hydra is rigorously maintained (*5*). For example, when a hydra is bisected transversely, the apical end always regenerates a base, and the basal end regenerates a head (mouth and tentacles). As another illustration, rarely does an extra tentacle or a secondary head occur along the body column close to the head. Even a bud, with its head and tentacles, develops distant from the parental head. Should extraneous heads or tentacles occur along the body column, however, the animal soon regulates its form until those structures no longer appear there.

By definition, then, it would follow that any hydra whose organization is atypical has deviated from its normal polarity. Therefore, some major questions are: *a*) What factor (or, most likely, factors) is responsible for polarity? *b*) Can any of these factors be analyzed and hence quantified at the cellular and biochemical levels?

We would like to show you three ways in which we are investigating polarity in hydra. The first involves an experimental study of reversal of polarity in normal hydra. Such studies, though common in the hydra literature, might help elucidate which areas of the animal are critical in influencing polarity. Our second approach involves the use of a developmental nonbudding mutant of hydra that appears to have lost some of its ability to regulate its polarity. Through progeny derived from grafts of this mutant with normal hydra we obtain situations analogous to neoplasia. We conclude by describing results of some of our preliminary studies with reliable microbiochemical methods for investigating the view that biochemical gradients might account, in part, for polarity.

REVERSAL OF POLARITY IN NORMAL HYDRA

It is generally recognized that, compared to the polarity of other hydroids, that of hydra is rigorously fixed (*5*). Reversals of polarity are difficult to obtain experimentally except by such drastic steps as: *a*) grafts in which a whole section of an animal is reversed (*5–7*); *b*) temporary grafts of two species of hydra (*8*); or *c*) exposure of cut portions of the animal to chemical fractions isolated from masses of hydra (*9*).

We report here a situation in which the polarity of part of the animal is permanently reversed following a simple transection of the body tube. The rationale for this series of experiments is as follows: In the presence of hypostome (mouth area) and its adjoining ring of tentacles, another head (hypostome and tentacles) or other tentacles are never observed to develop close by. For example, new buds with tentacles develop at about one-half to two-thirds the distance down the body tube. Possibly at this distance any influence (control, dominance) of the head is attenuated and a bud can form. Moreover, if in a nonbudding animal the lower part of the animal is removed, the cut section of the portion containing the head always regenerates a new basal disc rather than another head. The bottom half, on the other hand, in the absence of a head, proceeds to regenerate a new one. Such is not the case with all hydroids. If, for example, a specimen of *Corymorphora* is transected close to the head area, then the isolated head "regenerates" a second head where a base would be expected to develop (as in the hydra), a bipolar animal resulting (*10*). A similar bipolar regeneration is also observed in *Tubularia larynx* when a short piece of the hydrocaulus is isolated (*11*).

From such experiments it can be assumed that in hydra the head somehow regulates the adjoining area to prevent tentacle formation, although the tissue closest to the head has the greatest potential for tentacle formation, once the head is removed (*12*, *13*). Such concepts are not new; they have recently been restated and supported experimentally by Webster and Wolpert (*14*).

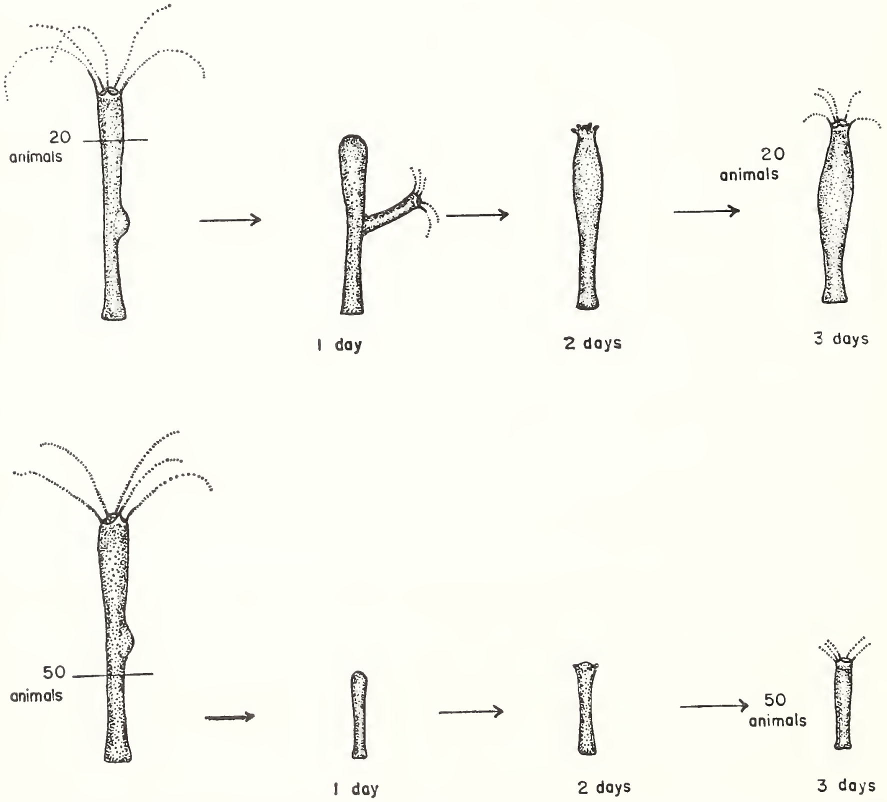
From such observations and any others like them, it seemed reasonable to suppose that a newly differentiating bud with the potential to develop a head may also inhibit tentacle development (as it occurs by regeneration or budding) in neighboring parental tissue. The structure of a budding animal permits this hypothesis to be tested by transection of the parental column at various levels above and below the bud. This experiment is not new. In fact, Trembley himself noted that the presence of a bud retarded the regeneration of tentacles on the amputated parent [*see* (*15*)]. Rulon and Child (*16*) also reported that the presence of the bud "... may inhibit reconstitution in the parent for as long as it remains attached if it is near the plane of section."

This part of our report gives the results of similar experiments in which budding hydra have been transected at different planes of the parental column. We found that the regeneration of a head on the parental column is inhibited in many cases following transection just distal to a beginning bud, even though the bud matures and detaches normally. Furthermore, a large percentage of such nonregenerating parental body columns develops a new *lateral hydranth* below the distal cut end of the remaining animal. Lastly, we show that when the distal nonregenerated portion of the animal with a lateral hydranth is removed, a head regenerates at the proximal rather than distal end, thus indicating a complete reversal of polarity of that portion of the animal.

Experiments and Results [6]

Control experiments.—As expected, animals without buds, when transected halfway between the head and basal disc (just above a potential budding zone), regenerated normally. That is, the distal sections developed functional basal discs within two days, and the proximal sections each possessed a hypostome with a ring of well-developed tentacles.

As another set of controls to the main experiment (text-fig. 2), 20 animals were transected halfway between the parental hypostome and the bud (upper series in text-fig. 1). Following the transection, the buds completed their development and detached at the normal time, but regeneration of the distal end of the parental column was retarded about one day, in contrast to regeneration of the distal end in animals without buds.



TEXT-FIGURE 1.—Effect of transecting hydra above the bud (*upper row*) and below the bud (*bottom row*) on regeneration of the parental column.

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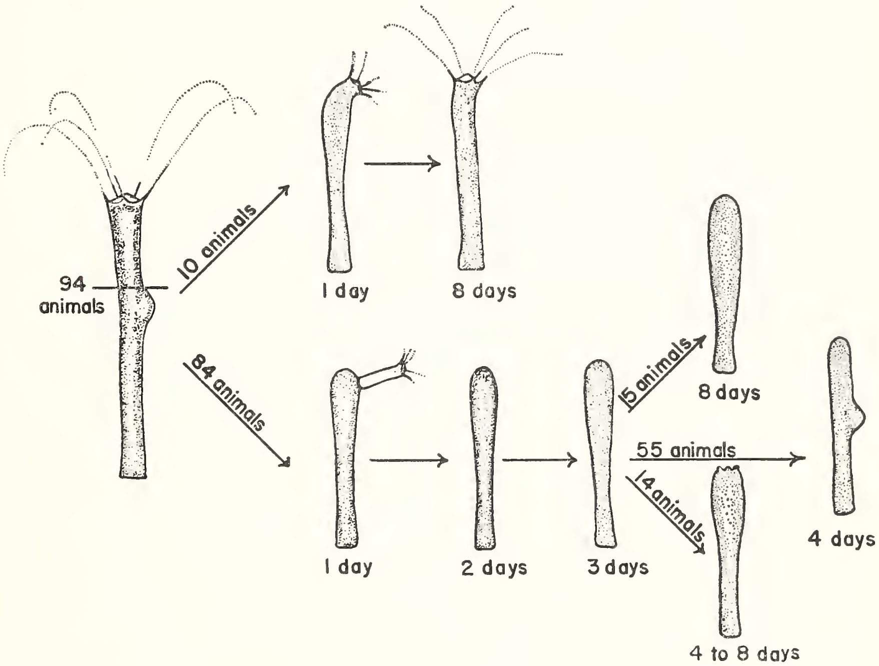
[6] All animals, *Hydra littoralis* (Hyman), were grown as previously described (*17*), and were fasted one day before experimentation.

A similar result was obtained in a second series of controls in which 50 animals were transected some distance below the bud (lower series in text-fig. 1). The basal sections of the parental columns all regenerated, but also at a slightly retarded rate.

Transection just distal to bud—formation of lateral hydranth animals.—Strikingly different results were obtained, however, when animals were sectioned just distal to their beginning buds so that the basal pieces bore those buds just below the plane of section (text-fig. 2). When 94 hydra were sectioned in this manner in one series of operations, the basal portions bearing the buds were observed to develop as follows:



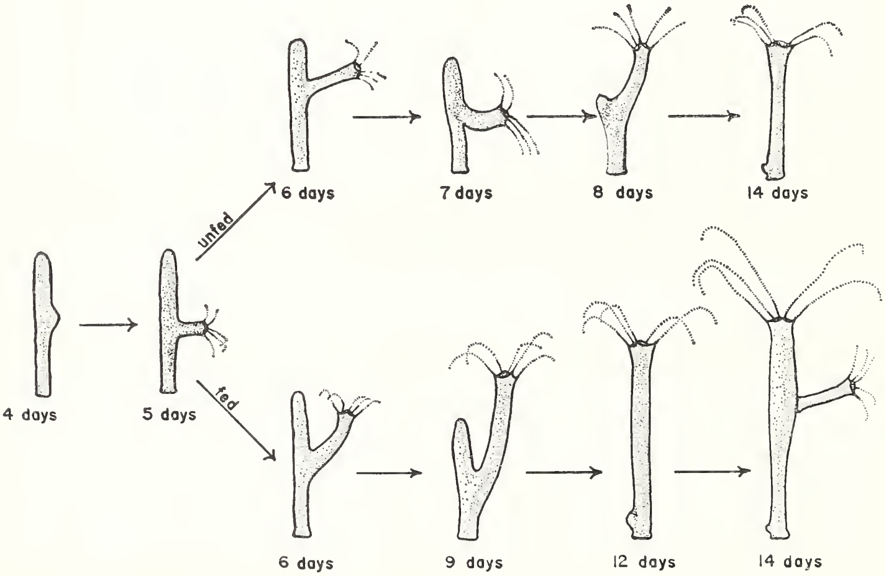
Text-figure 2.—Production of lateral hydranth animals by transection of parental body column immediately distal to a beginning bud.

In ten instances the bud developed a normal hypostome and tentacles, but failed to detach from the parental column. Instead, the bud remained attached to it, and gradually the acute angle between bud axis and parental axis lessened until the bud replaced the distal portion of the parental body, without regeneration occurring at the plane of section. In a few cases a small basal disc differentiated on the parental column near the base of the bud and persisted for some time.

In the remaining 84 cases, the buds differentiated normally and detached 2 or 3 days after the operation. Of these 84 parental body tubes, 15 gave no indication of any regeneration for the 8 days they were observed after the operation. (In another series of similar experiments, regeneration did not occur in the same type of animal during the 21 days they were under observation.) Between the 4th and 8th days, 14 other animals gave feeble signs of regeneration and formed hypostomes with short tentacles.

The basal portions of the other 55 animals (59%) developed in an unexpected and bizarre manner. Between the 4th and 7th days after operation, they all formed a small protuberance about halfway between the original site of sectioning and the basal disc (text-fig. 2). Within a day or two these protuberances developed hypostomes and 2 or 3 tentacles (text-fig. 3), and began elongating to form short columns at right angles to the parental axis. Animals of this type will be referred to hereafter as *lateral* hydranth animals.[7] Such animals were usually obtained about 50–60% of the time whenever the same kind of transection was performed.

Development of lateral hydranth animals.—The development of lateral hydranth animals is shown in text-figure 3. In one group of animals not fed after development of the lateral tentacles and hypostomes, the lateral axis gradually increased in length while the remaining parent body tub became relatively smaller and gradually (within 3 weeks) disappeared at the basal disc. Within a few days the lateral axis had essentially replaced the parental column, a remnant of which remained as a small protuberance near the base of the animal. Similar results were obtained with a number of animals fed via the lateral hydranth. These animals increased in total size, and buds developed on the "new" column and



TEXT-FIGURE 3.—Developmental fate of lateral hydranth animals maintained without food (*upper row*) and with food (*bottom row*).

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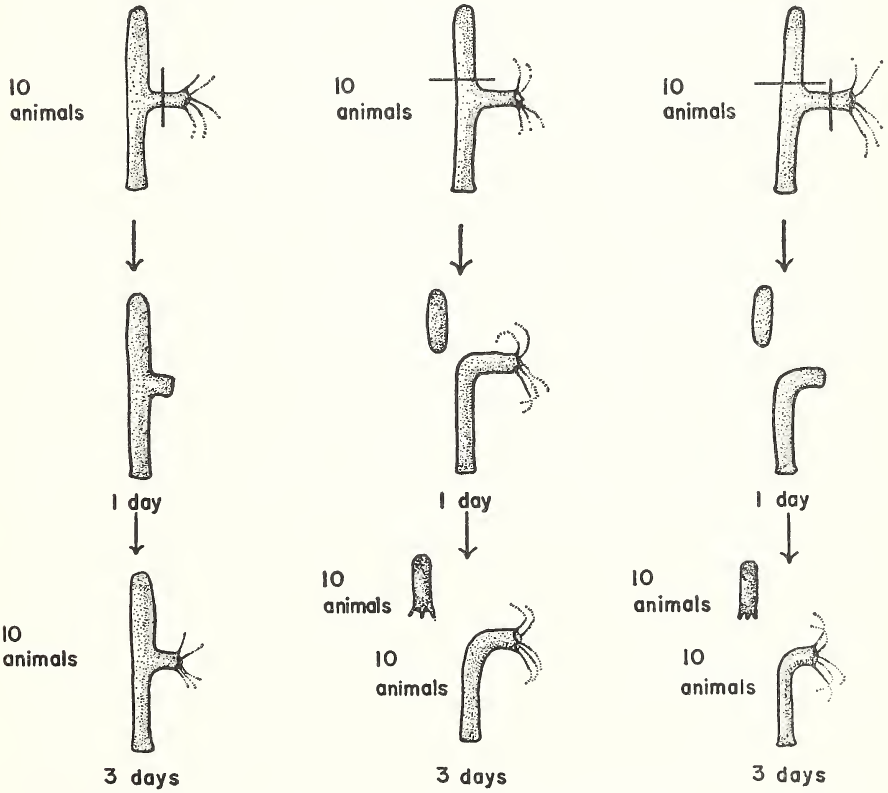
[7] These lateral hydranths can in no way be considered as buds. Only full-size hydra produce buds. Buds form in a specific manner, complete their development, and detach, all in about 2 days. Buds seldom form in hydra starved for 4 days.

detached in the normal manner. However, just as when the lateral hydranth animals were not fed, the remaining parent body tube became smaller, gradually disappearing at the basal disc.

Regeneration of animals with lateral hydranths.—When the hypostome and tentacles of 10 lateral hydranth animals were removed, within 3 days a new hypostome and tentacles regenerated at the site of the section (text-fig. 4, first col.). On the other hand, when 10 lateral hydranth animals were sectioned through the main parental tube just above the point of attachment of the lateral hydranth, no regeneration occurred at the site of section (text-fig. 4, second col.). Instead, in every case the small part of the parent column that had been removed regenerated a hypostome and set of tentacles *at the site of section*, not at the end from which the parent hydranth had been previously removed (text-fig. 4, center col.). In addition, the former apical end developed a functional basal disc. This result clearly shows that the polarity was reversed in the distal portion of the lateral hydranth animal.

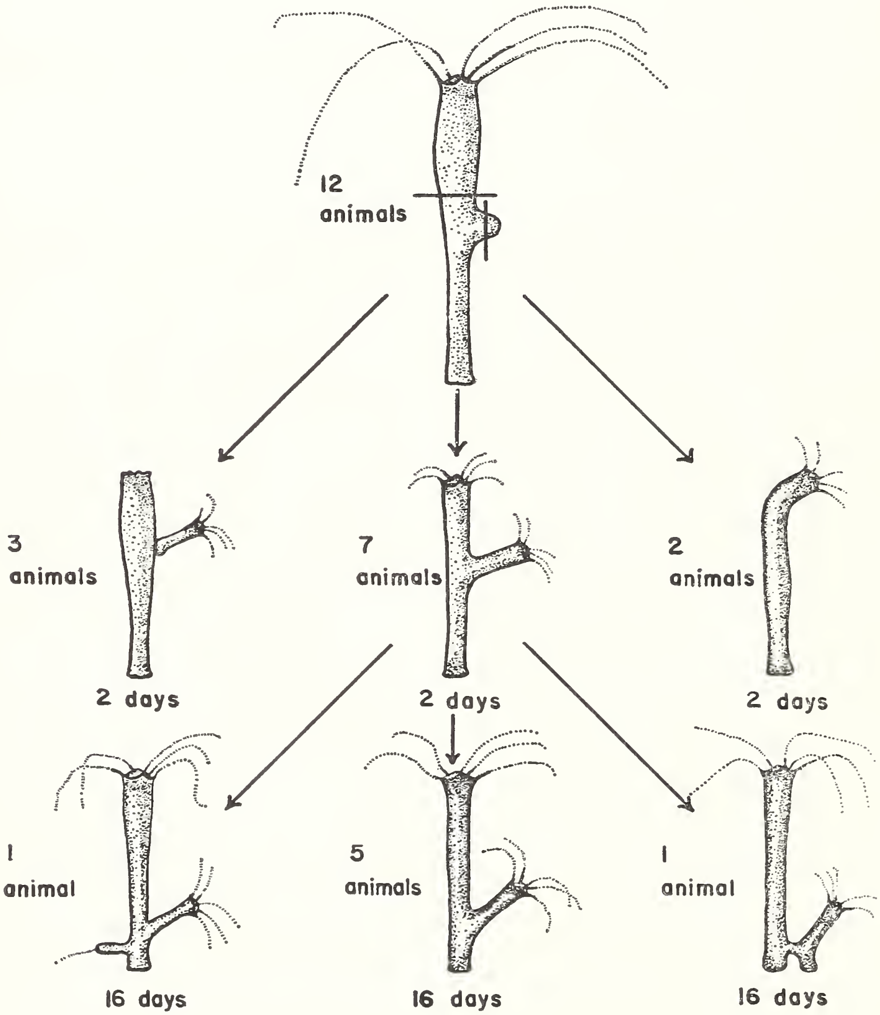
Similar results were obtained with a third group of 10 lateral hydranth animals transected both below the head of the lateral hydranth and across the parental axis just apical to the point of attachment of the lateral column (text-fig. 4, last col.). In these cases the lateral columns regener-



TEXT-FIGURE 4.—Regeneration patterns of lateral hydranth animals.

ated new tentacles and hypostomes; the small pieces of parental column regenerated tentacles at the site of section, *i.e.*, with reversed polarity as compared with the polarity of the original parental body tube. In addition, as illustrated in text-figure 4 (center col.), the small polyps with reversed polarity also developed functional basal discs.

Inhibition of lateral hydranth formation by an additional transection.—The experiments discussed thus far indicate that lateral hydranth animals developed only when a normal bud was present on a parental column transected just distal to that bud (text-fig. 2). Thus the removal of that bud should prevent the formation of lateral hydranth animals. Such proved to be the case (text-fig. 5).



TEXT-FIGURE 5.—Inhibition of formation of lateral hydranth animals by removal of tip of beginning bud at the same time the parental body column is transected.

Twelve animals with beginning buds were sectioned just proximal to the tip of the bud and across the parental column just above the point of bud attachment (text-fig. 5). In 2, the buds regenerated heads (in about 2 days) but did not detach from the parents; instead they gradually replaced the section of the parent removed by the operation. In 3, both the parents and buds regenerated heads. The buds then completed their development but migrated down the parental column an appreciable distance before detaching (recall that a new head will not regenerate close to another one).

In the remaining 7 animals, both the buds and the parents regenerated new heads, but the buds did not detach during the 16 days they were observed. These new hydranths (which at one time had the potential to become buds) remained attached to the parental tube and led to the formation of unusual H- and Y-shaped animals. Gradually these new hydranths moved toward the parental basal discs.

Thus, on removal of the apical tip of the bud simultaneously with transection of the parental tube just distal to the bud, the remaining parental body tube was able to regenerate a new head.

Discussion of Polarity Experiments

An "operational" summary of these experiments would seem rather simple. That is, the presence of a beginning bud on an animal transected just distal to that bud leads to the formation of a lateral hydranth animal. In this animal, normal regeneration is inhibited, the polarity of the distal part of the main body column is reversed, and a new lateral center of polarity develops.

At this point, however, we come to a standstill. We are forced to impart mysterious powers to the tip of the bud. Does it prevent regeneration of the cut section of the parental tube? And if so, how? By releasing an "inhibitor" (or removing a "stimulator")? By acquiring critical cells (such as the interstitial cells) from the parental body column? Are the patterns of cell division altered? To my mind, despite the large number of experiments on regeneration, grafting, and reversal of polarity, and despite the varied theories with respect to gradients of stimulators and inhibitors, there exist no satisfactory interpretations of the developmental phenomena observed in hydra. Abraham Trembley described most of these phenomena over 200 years ago in his remarkable *Mémoires* (*18*), but concluded: "There is no better way to explain the facts we know than by trying to discover new ones." His statement holds true today as it did then.

I have no answers to explain the mechanisms accounting for the experimental results just described. But perhaps no answer is a good answer because it leaves the way clear to search for new facts. Thus, to provide more facts on these most basic questions, I now turn to a mutant of hydra which has an atypical polarity and seems to have lost its ability to regulate its development in the rigorous ways of the normal animal.

A HYDRA MUTANT—ITS ATYPICAL POLARITY AND REGENERATIVE CAPACITIES, AND ITS USE AS A POSSIBLE NEOPLASIA ANALOG

Through work on the regenerative properties of a mutant hydra, we encountered some phenomena that provided us with an experimental approach to the study of a possible neoplasia analog. These experiments may give us insight as to how neoplastic growth may have developed among organisms of this most primitive phylum.

Before presenting the data on these analogs, however, I first will describe some unusual developmental properties of the mutant and will point out how this animal does not show the inflexible polarity (*i.e.*, the control of form) of normal hydra.

Regenerative Capacities

In the past it had been difficult to find mutants of hydra, because it was not possible to secure sufficient hydra undergoing sexual reproduction and thus to provide a supply of zygotes, among which mutants could be sought. In recent years, however, methods have become available for raising large numbers of animals in the laboratory (*2*, *3*) and for inducing those hydra to produce gametes (*19*, *20*). From mass cultures of *Chlorohydra viridissima* (Florida strain, 1961) we isolated a number of encysted zygotes, out of which we hatched hydra having morphological aberrancies. One such animal was particularly unusual in that *a*) it did not produce asexually by budding; *b*) in comparison with normal hydra, its size, dry weight, and protein content were about ten times greater (*21*) ; *c*) it could not be induced to form gonads under conditions in which gonads are induced in the normal animal; *d*) it often took on multipolar shapes; and *e*) these multipolar shapes arose particularly from regenerating animals. These regenerative properties of the mutant highlight aberrancies in the organization of the animal.

As indicated in the previous section, when a normal hydra was bisected, the apical portion regenerated another basal disc while the basal portion regenerated another head. Such is not the case with this mutant. The apical portion regenerates[8] a second head, giving rise to a bipolar animal (fig. 1).

Even stranger is regeneration in the basal portion of the mutant. First, as in a normal hydra, another head regenerates at the site of the section; at the same time numerous small protrusions being to occur, as if at random, along the bottom part of the remaining animal (fig. 2). Each protrusion then develops into a tentacle. Often totaling over 50 in number, these tentacles cluster in groups of 6–10 (fig. 3). In the center of each cluster a mouth and hypostome develop, and from these many small hydranths emerge along the body tube (fig. 4). Each of these hydranths is capable of feeding. After about 2–3 weeks, each hydranth appears as a complete animal still attached to a central stem (fig. 5) in a manner reminiscent of a colonial marine hydroid. During the subsequent 2–3 weeks, each hydranth separates as a monopolar animal.

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[8] The term "regeneration" does not apply strictly here because regeneration means to replace a lost part. In the apical portion of the mutant, it does not replace the lost part but rather duplicates the transected apical end. Hence the term "heteromorphosis" more properly applies.

Thus, in the manner described, these mutants not only regenerate, but through an atypical regeneration, are able to increase in number. This unusual type of asexual reproduction also occurs in mutant hydra that have not been transected. Shown in figure 6 are all the offspring that developed from a single monopolar mutant animal over a 3-month period. Various phases of the stages just described in the regenerating animals can be seen among these animals.

Two things are to be kept in mind when interpreting the development of the animals shown in figures 2, 3, and 4. First, the asexual reproduction observed in the mutant is not the budding process that takes place in the normal hydra. Budding is a specific and rapid event. First a protuberance forms at a specific site along the parental body column. Then a ring of tentacles and a mouth develop at the tip of this protuberance, and finally a basal disc develops and the bud detaches. This entire process is highly organized and takes about 36 hours (*see also* footnote 7).

Secondly, the random formation of tentacles and new hydranths seen in figures 2, 3, 4, and 6 never occurs in that part of the animal close to the distal head. This region usually makes up from one-fourth to one-third the tissue of the body column of the mutant animal.

Is there anything peculiar about this region of the body column of mutant animals? Because this region gives a semblance of having normal organization, is it more like the body tube of the normal hydra than is the rest of the body tube? Dr. Richard Campbell and Mr. R. Tinsley of the University of California at Irvine believe so (personal communication). In some clever and simple experiments they showed that the apical portion of the mutant will regenerate as do normal hydra if the mutant is transected close to the head. For example, they showed that the apical portions of normal *C. viridissima*, regenerating body columns of 0.5–2.0 mm in length developed basal discs 100% of the time. The apical portion of the mutant also developed basal discs 100% of the time only when the regenerating body column was 1.0 mm long or less. When the regenerating body column of mutant animals was 1.5 mm long, then only 88% developed basal discs while the others developed a second head. When it was 2.0 mm long, then about half formed basal discs and half formed another head. Mutants with regenerating apical body columns much greater than 2 mm in length always formed a second head, as we have observed innumerable times.

It is important to point out that the situation observed by Campbell and Tinsley is completely different from that observed with *Corymorphora* (*10*) and *Tubularia* (*11*). These latter hydroids form more bipolar animals, the closer the transection is to the head, whereas the mutant hydra forms more bipolar animals, the farther the transection from the head.

How can we interpret these findings? Again it is difficult to do so without imparting mysterious properties to the apical end of the mutant. Perhaps the head end in some unknown manner controls the polarity of hydra. In normal hydra this control appears to extend over the entire animal, whereas in the mutant it extends over only part of the apical portion of the animal. But because the mutant is so much larger in volume and length than the normal animal, possibly the task of controlling polarity in large mutants becomes too great, especially over long distances. Hence the atypical regenerative properties of the mutant and the atypical organization of the basal portion of the animal may merely reflect pleomorphic effects owing to the large size of the animal. This large size probably comes about because, although the animal feeds, it is unable to channel this growth into the formation of buds; instead, the mutant increases into a size that it cannot regulate. Thus the key to determining the cause of many abnormal properties of the mutant may lie in uncovering a defect in the budding process.

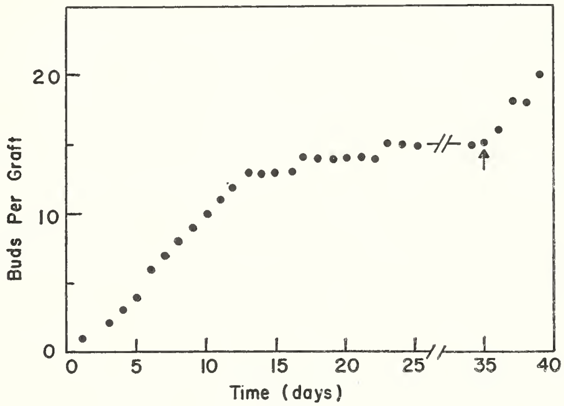
A Neoplasia Analog

Experiments with the mutant hydra led to an investigation of what may be considered a primitive type of neoplasia. In essence, because permanent grafts between the mutant hydra and normal individuals of the same species could be made, it was possible to derive organisms having populations of cells of different genotype. These individuals we call "heterocytes," borrowing a term from Filosa (*22*), who applied the name to slime molds made from populations of normal and mutant cells of the same species.

Our first heterocytes were found as a result of the following experiment. We were perplexed as to why the mutants were not able to bud. They did not seem to be deficient in any nutriment; even animals reinfected with algae—hence able to derive extra nourishment therefrom (*23*, *24*)—and fed to repletion daily, were not able to bud. To determine whether it might be possible to transfer some factors (macromolecules, cells, intercellular particles?) present in normal individuals to mutant hydra, and thus induce budding, a series of grafts was made. When the apical half of the graft came from a mutant hydra and the basal half came from a normal individual, budding was initiated in the mutant portion within 1 day and continued at a near constant rate for 13 days (text-fig. 6). The budding process appeared normal, and all the progeny were like the mutant; that is, they became large and did not bud. Following some sporadic budding after day 13, budding ceased by the 23d day after the graft was made. On day 35, the basal half of the experimental animal was removed, and another basal portion from a normal hydra was grafted there. Again budding was initiated in the mutant portion of the animal, but in contrast to the progeny initiated by the original graft made 35 days earlier, some of the new progeny appeared mutant, whereas others appeared normal.[9]

These normal-looking buds were of the same morphology as that of normal animals, were of the same size, and proceeded to bud rapidly when fed more *Artemia* nauplii. Yet their buds also retained some properties of the mutant for they, like the graft after day 35, proceeded to bud both normal and mutant-appearing individuals. For example, 5 of these heterocytes (which resembled normal hydra) were placed singly in separate petri dishes, and each was allowed to reproduce, by budding, a group of about 100 hydranths.[10] The number of hydranths in each colony increased at a logarithmic rate, and the doubling time (*25*) was measured (table 1, col. 1). Among the groups derived from each heterocyte were a number of animals having the characteristic morphological and developmental properties of the mutant (table 1) and a greater number that appeared normal.

This experiment shows, first, that it is possible to obtain, through budding of a single heterocyte, progeny of two distinct phenotypes. Thus mutant somatic cells appear to proliferate in a host "normal" individual, to segregate, come off as a bud, and express their phenotype in those buds. Secondly, the groups with the largest number of mutants had the slowest doubling times. On the other hand, those colonies having doubling times



Text-figure 6.—Induction of budding in the apical half of a nonbudding mutant hydra by grafting to it the basal half of a normal (aposymbiotic) specimen of *C. viridissima.* The first graft was made on day 0, a second on day 35.

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[9] The induction of budding in mutant grafts from normal animals always occurred, although it did not follow exactly the pattern shown in text-figure 6. Sometimes the initial graft induced budding (and the production of heterocytes) for as long as 40–60 days. Possibly the extent to which budding and the production of heterocytes were induced depends on the relative sizes of the mutant and normal grafts.

[10] A hydranth as used in this context is described in (*26*).

TABLE 1.—Production of "normal" and "mutant" progeny by budding of 5 heterocytes of normal appearance

T*	k†	Progeny		Progeny as mutant (%)
		Normal	Mutant	
1. 60	0. 43	115	1	0. 87
1. 65	. 42	126	8	5. 97
1. 80	. 39	132	9	6. 38
1. 90	. 36	57	9	15. 8
1. 95	. 35	50	19	27. 6

*The term T refers to the time, expressed in days, in which the numbers of hydranths (*26*) doubled in number [Reprinted from (*21*) with permission of *Science*.]

†The clonal logarithmic growth rate k was obtained by using the equation for logarithmic growth (*25*): $k=ln\ 2/T$.

close to those of normal symbiotic and aposymbiotic *C. viridissima* (approximately 1.5 days) gave rise to few mutant heterocytes. Perhaps both the slower growth rate and the large proportion of mutants among the progeny reflect a greater number of mutant cells in the parent organism.

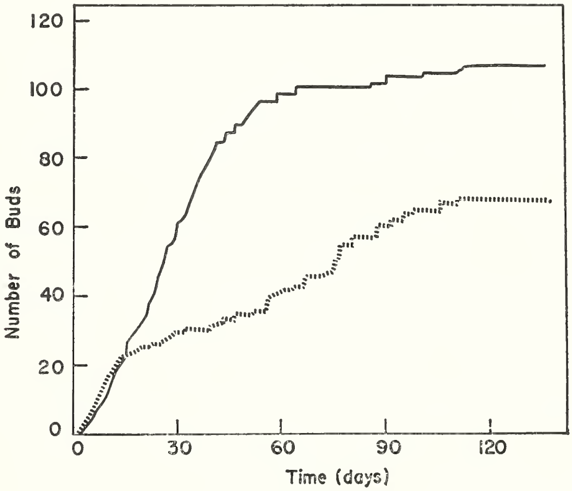
From the evidence on hand, it is not possible to determine whether the budding of a mutant-like hydra by a normal-appearing animal resulted from a specific migration of populations of mutant cells into the budding zone, or whether it was just by chance that a large number of mutant cells were in the budding region.

Because each of the normal-looking progeny produced in such an experiment (table 1) could bud both mutant and normal heterocytes, a major practical benefit ensued from these findings. By growing large numbers of hydra derived from normal-appearing heterocytes, we were able to harvest "mutants" in vastly greater numbers than would be possible through increasing the clone by means of the tedious bisecting procedure. A culture of such "derived" mutants is shown in figure 7. It would appear as though the mutant cells which normally are difficult to obtain in large numbers because of the inability of the mutant to bud, can be obtained in great numbers through the vehicle of the heterocyte. In essence, the "normal" hydra acts as a "tissue culture" for raising mutant cells. This procedure for "rearing" mutants has also been applied successfully in this laboratory with another kind of nonbudding hydra mutant of the same species.

Can we consider the phenomenon observed in the normal-looking heterocytes as analogous to neoplasia in higher organisms? In these hydra we have a situation in which populations of somatic cells of a different genotype grow and accumulate in a host organism. Eventually a mass of these cells forms, but rather than produce an abnormal growth as in higher animals, the cells mold into a bud and leave the host in the same manner as do normal cells. Because hydra do not have complex vascular and nervous systems, there is little chance of masses of these cells obstructing any critical areas.

There is one way, however, in which the mutant cells in the normal-looking heterocyte can deleteriously affect the host. Whenever we reared one of the animals for 2 weeks to 5 months, it invariably changed its morphological and reproductive characteristics and became gradually transformed into an animal of mutant phenotype. Never has a polyp of mutant phenotype been observed to transform into one of normal phenotype.

Experiments illustrating the transformation of a heterocyte of normal appearance into one of mutant phenotype were carried out as follows. As a daily procedure, 2 normal-appearing heterocytes were fed to repletion with *Artemia* nauplii, their culture solution was changed twice, and the number of buds they produced was recorded. Both animals budded at a normal rate for at least 2 weeks (text-fig. 7). At that time 1 animal (*dotted line*) became slightly larger and started to take on the appearance of a mutant. This transforming animal continued to bud, but at a slower and irregular rate. After a month, budding ceased and the once normal-appearing heterocyte was gradually transformed, like the mutant, into a multipolar animal. This multipolar animal then slowly divided into 6 monopolar mutant-like animals, a few of which sporadically gave off a number of small, budlike individuals. After 100 days virtually all "bud" production ceased, with the mutant phenotype finally dominating the resultant monopolar animals.



Text-figure 7.—Budding rate of two heterocytes resembling normal hydra. (Reprinted with permission of *Science*.)

The other heterocyte (*solid line*) continued to give off buds at a constant rate for about 40 days before it began undergoing a similar transformation. The monopolar mutant-like individuals derived from this normal-appearing heterocyte showed fewer latent bursts of budding activity than did the individuals derived from the other.

These transformed hydra, like any hydra fed and cleaned often, did not die over the period of observation (sometimes up to 2 years). Yet we can say that the mutant cells, by spreading through these once normal-appearing individuals, brought about the reproductive death (*i.e.*, by budding) of their host organism.

These experiments are but 2 of more than 25 conducted, in which the normal-appearing heterocytes always became mutant. The rate at which this transformation took place probably depended on the number and location of mutant cells in the original heterocytes. The mechanism underlying this transformation remains unknown.

Discussion

The results of these experiments (table 1, text-fig. 7) demonstrate two properties of mixed population of somatic cells: cellular segregation and heterocyte dominance. These two phenomena, as they occur in hydra, may have broad biological significance. For example, with respect to development, we have a system whereby cells of one set of inheritable characteristics can exert their developmental propensities over those of another and can be expressed in the phenotype of the animal. With reference to evolution, the eventual dominance of mutant somatic cells may play a major role in the evolution of metazoan species reproducing solely or primarily asexually. Lastly, the hydra heterocyte allows us to study a system in which mutant somatic cells can spread throughout an animal and eventually dominate the host tissue in a manner analogous to like processes of some tumors.

With reference to this last point, although the normal-appearing heterocyte with its mutant cells arose from grafting experiments, possibly a similar situation could arise naturally. That is, should spontaneous mutations of somatic cells occur in a normal hydra, these mutant cells might also be able to proliferate, segregate, and dominate the host animal. Should these processes occur spontaneously in nature, then there is represented in hydra a situation analogous to that of neoplasia. Whether they do or not, the system described herein offers us an opportunity to study the influence of mutant somatic cells on adjacent normal tissue. Hydra offer an especial experimental advantage for this type of work in that, unlike most other metazoa, hydra can be made to grow continuously and to reproduce solely by asexual means. Consequently, the mutant somatic cells do not die with the host, but are distributed indefinitely among the progeny where interactions with normal cells may be allowed to express themselves.

Lastly, this nonbudding giant mutant may help us to understand polarity in normal hydra and to investigate mechanisms of budding and its induction.

MICROBIOCHEMICAL ANALYSES FOR GRADIENTS OF ENZYME ACTIVITIES

It is becoming increasingly apparent that in order to understand growth and polarity in both the normal and mutant hydra, we must broaden our approaches to include techniques for investigating cellular and chemical interactions. A great stimulus was given to a chemical study of polarity in coelenterates by C. M. Child about 50 years ago. He proposed that development was initiated and controlled by the region of an organism having the highest metabolic rate [*see* (*10*)]. His metabolic gradient theory, first proposed from experiments in regenerating *Tubularia*, was supported by later experiments in which hydra showed an axial gradient in their susceptibility to poisons (*27*) and their ability to reduce methylene blue that had previously been taken up by their cells (*28*). In addition, the oxygen consumption of isolated pieces of living *Tubularia* indicated an apical-to-basal gradient of activity (*29*).

More recently the gradient theory has been tested by histochemical techniques. For example Haynes (*30*), using whole hydra, attempted to use results from a histochemical assay for succinic dehydrogenase activity as a measure of a metabolic gradient. He found that the pattern of the localization varied, depending on the species of hydra used. Lentz and Barrnett (*31*) also measured succinic dehydrogenase histochemically but found no localization of activity.

The analytical methods used to gather information that supports the gradient theory are qualitative and fraught with analytical artifacts. For example, the results obtained by the use of dyes and poisons may be partially caused by the different permeability of specific areas of the animals to those substances. Furthermore, differences in respiration observed in small sections of living tissue may result from increased respiration associated with a wound response to the cutting procedure.

Gradients shown by histochemical methods may also be misleading. For example, the problem of permeability to the histochemical reagents exists. In addition, it is difficult to quantify the activity of many enzymes by histochemical means. Also, unless the *p*H optimum and K_m of the enzyme have first been determined on extracts of the tissue, it is difficult to know if the assay was carried out at optimal conditions.

The approach we are now beginning to use to study the localization in hydra of enzymes, substrates, and coenzymes does not have any of the handicaps of the earlier methods. In essence, our methods are a modification of the ultramicrochemical techniques developed by Lowry and his associates (*32*) for the study of the localization of enzymes, substrates, and pyridine nucleotides in various regions of the mammalian brain. First we analyzed extracts of hydra for the properties of the enzyme under question. That is, we determined the K_m for the substrates and coenzymes of the enzyme, the *p*H optima, and possible metal requirements. These routine enzymological experiments were necessary because enzymes of hydra might not be expected to have the same properties as mammalian or bacterial enzymes. For example, we found the nicotinamide adenine dinucleotide phosphate ($NADP^+$) isocitrate dehydrogenase (ICDH) to have a 100-fold higher K_m for isocitrate than did ICDH from hog heart. Thus, if levels of isocitrate close to the K_m of ICDH from mammalian tissues were used to measure ICDH in hydra, little or no activity would have been found.

The second Lowry procedure we used involved the preparation of freeze-dried hydra. By freezing our animals instantaneously with liquid Freon chilled in a dry ice-acetone bath, and then drying the animals at −20°C, we were able to obtain hydra in which the levels of enzymes or substrates assayed differed negligibly from the live animals. By hand-dissecting 0.1–0.8 μg bits of dried hydra, we were able to investigate for the distribution of enzymes anywhere along the body of a hydra. In an average experiment, 30–50 pieces were removed from a single animal. It was important to use fragments this small for another reason: Using larger pieces (2–5 μg) we found that all the enzymes did not diffuse out into the reaction mixture.

There were three other practical advantages of using freeze-dried hydra: *a*) Before the enzyme was assayed for, the dried tissue could be weighed on a quartz balance; hence it was possible to get the specific activity on a dry-weight basis for each piece of tissue analyzed. *b*) By cutting pieces of tissue from different levels of dried hydra, we eliminated all wound artifacts possibly coming from cutting the live animal. And last but not least, *c*) more accurate dissections could be made on the dried hydra because they did not squirm.

The third Lowry procedure we used was that of analyzing for enzyme activities fluorometrically. This method applies to enzymes that either reduce or oxidize the pyridine-nucleotide coenzymes. Fluorometric methods can detect these coenzymes or their derivatives at about one thousandth the concentration measurable by spectrophotometric means. The method can also be adapted to many enzymes that do not react directly with a pyridine nucleotide because it is usually possible to couple it with an auxiliary enzyme so that the oxidation or reduction of pyridine nucleotides can be measured.

Hence, by modifying these procedures, the sensitivity for our enzyme assays was greatly amplified and it was possible to measure the distribution of enzyme activities using a single hydra. Our exact methods will be presented in a forthcoming paper (*33*).

Localization of Two NADPH-Producing Enzymes

Our first analysis concerned the localization of two NADPH-producing enzymes. One was glucose-6-phosphate dehydrogenase (G6PDH), the first enzyme in the hexose monophosphate shunt. The other was isocitrate dehydrogenase (ICDH), an enzyme in the citric acid cycle complex. The

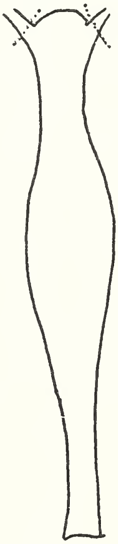
NADPH generated by these reducing enzymes is considered as being used particularly in fatty acid synthesis.

Measurements made on more than 300 pieces of dried hydra from 32 animals revealed that G6PDH was 20–100% more active in the area of the hypostome and base than in the midportion of the hydra. Conversely, the specific activity of ICDH was 20–200% higher in the midportion of the animal than at the hypostome or base. In most hydra analyzed, the ICDH activity of the hypostome was lower than that in any other part of the animal.

Text-figure 8 shows the results when we took two specific examples and normalized the specific-activity measurements. All activities were expressed in relation to the activities in the midportion of the animal, which were relatively constant and set at 1.00. These results agree with a larger number of samples which show that G6PDH activities of both the hypostome and base were significantly different ($P > 5\%$) from the activity of either midsection. There were also significant differences between the ICDH activity of the base and either midsection, and between the activity of the hypostome and the midsection nearest the base ($P > 5\%$). The dif-

Relative Specific Activities

G6PDH	ICDH
1.97	0.66
1.31	0.91
1.86	0.99
1.27	1.00
1.23	"
1.00	"
"	"
"	"
"	"
"	"
"	"
"	"
"	"
"	"
"	"
"	"
"	"
1.15	0.91
1.38	0.66
1.44	0.67



TEXT-FIGURE 8.—Relative specific activities of glucose-6-phosphate dehydrogenase (G6PDH) and isocitrate dehydrogenase (ICDH) in the body column of specimens of normal nonbudding *Hydra littoralis*. The first number in each column represents the relative specific activity in the hypostome.

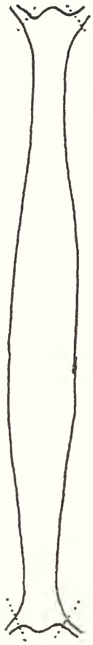
ferences between the activities of the midsections for either G6PDH or ICDH were not significantly different ($P>5\%$).

Distribution of Enzyme Activities in Bipolar Forms of a Mutant Hydra

Similar assays were made of bipolar forms of the mutant hydra (*i.e.*, forms having a head at both ends) to determine if the unusual polarity of the animal was expressed in the patterns of distribution of the enzymes. About 150 measurements were made on 6 animals. All animals were those forms without endosymbiotic algae. The data, shown in text-figure 9, were normalized and expressed as in text-figure 8. The distribution of G6PDH in such bipolar animals (text-fig. 9) was similar to that found in the normal *C. viridissima* (without algae) and in *H. littoralis* (text-fig. 8), *i.e.*, the highest activity occurred at the extremes of the body column. The ICDH activities in the mutant, however, did not follow the pattern found in normal monopolar animals. Instead, there was more random distribution of activities with no apparent localization.

Relative Specific Activities

G6PDH	ICDH
1.68	0.96
1.41	1.04
1.18	0.97
1.14	0.62
1.00	0.66
"	1.00
"	"
"	"
"	"
"	"
"	"
"	"
"	"
"	"
"	"
"	"
"	"
"	"
"	0.86
"	0.78
1.07	0.92
1.04	1.03
1.58	0.88



TEXT-FIGURE 9.—Relative specific activities of glucose-6-phosphate (G6PDH) and isocitrate dehydrogenase (ICDH) in the body column of a specimen of a bipolar mutant of *C. viridissima* (aposymbiotic). The first and last number in each column represents the relative specific activity in the hypostomes.

Conclusions and Summary

We are now able to apply microbiochemical methods to analyze the localization of enzymes along the body column of hydra. The results from these analyses are free from possible artifacts inherent in other methods used previously to show the distribution of metabolic activities in these animals.

Our results show that the NADPH-generating enzymes are localized in hydra, with G6PDH particularly high and ICDH low, in the hypostome and base regions. Hence, specific areas along the body column have the potential for producing NADPH at different rates. For such a potential to be realized, however, the level of substrates and coenzymes must be present at saturating levels in all regions of the animal. Thus, differing rates of NADPH production along the body column could occur regardless of any enzyme localization, depending on the concentration of substrates and coenzymes at each specific area.

Although we show localization of two NADPH-producing enzymes in hydra, we cannot conclude that these enzymes are responsible for any specific role in affecting polarity, metabolic gradients, or development of the animal. The amount of enzymes can, however, affect the level of substrates present in the various regions. Thus, should the view hold true that the ratio of substrates to enzymes affects final gene expression (*34*), it would be valuable to determine the concentrations of substrates and coenzymes in each area of the hydra. Toward such a study, we are presently adapting the enzymatic cycling methods (*35*) which can detect 10^{-12}–10^{-15} M concentrations.

One additional point. Most information gleaned from applying known methods to a new experimental animal is of interest in comparative biochemistry and physiology. This is especially true for studies of enzymes in hydra because *a*) coelenterates occupy a pivotal phylogenetic position in the evolution of multicellular organisms and, *b*) to date, only a few enzymes from coelenterates have been characterized. Besides finding that some of the K_m values for the hydra enzymes were high, relative to the K_m values of similar mammalian enzymes,[11] we also noted that even a metabolic pathway in hydra was not completely typical. Specifically, although hydra were particularly rich in G6PDH, the next enzyme in the hexose monophosphate pathway, 6-phosphogluconate dehydrogenase (6PGDH), was absent (*37*). We have shown further that 6PGDH is absent from all marine, freshwater, and brackishwater hydroids analyzed, as well as from most scyphozoans (*38*). Yet the enzyme is present in all other coelenterates (anemones, corals) assayed and at about the same level as G6PDH. A near 1:1 ratio of G6PDH to 6PGDH is common in most other multicellular animals having a hexose monophosphate pathway.

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[11] This finding may support the view of Kaplan *et al.* (*36*) that the molecular forms of some enzymes from invertebrates differ from those of vertebrates.

REFLECTIONS AND PERSPECTIVES

This being the first Symposium on Neoplasms and Related Disorders of Invertebrate and Lower Vertebrate Animals, a few historical notes may be warranted. Much of the present-day research on hydra and other coelenterates has as its greatest stimulus a quick succession of papers by W. F. Loomis dealing with culture methods (*2*, *25*), the glutathione-activated feeding behavior (*39*), and the CO_2 induction of sexual differentiation (*19*). Yet one of the original and major stimuli for Loomis to use hydra was his desire to investigate neoplasia in a relatively simple metazoan. I recall hearing in the spring of 1953 at the Johns Hopkins University, Loomis' first seminar on hydra. His last slide showed a coin: One side represented abnormal development, as seen in cancer, and the other side represented normal development. A proper understanding of either side of the coin, he pointed out, would lead to an understanding of the other. Loomis, like so many of the researchers on coelenterates, chose to investigate normal development. He focused most of his attention on the chemical factors leading to the transformation of the interstitial cells into gametes (*19*, *40*).

Much has happened in biology since 1953. Now it is common talk to say that "the next big push will be in developmental biology." It is apparent from the presentations given at this Symposium that research on the whole animal raises more questions than it answers. Research on lower forms new to modern research is especially in need of basic groundwork in cytology, biochemistry, and physiology.

There is a growing dissatisfaction among many investigators of the primitive metazoans with our limited understanding of normal development in coelenterates. If this then is the time for a surge in developmental biology, we welcome fresh approaches to help solve these fundamental problems of normal (and abnormal) development, most of which have been tantalizing biologists since Trembley published his *Mémoires* in 1744 (*18*).

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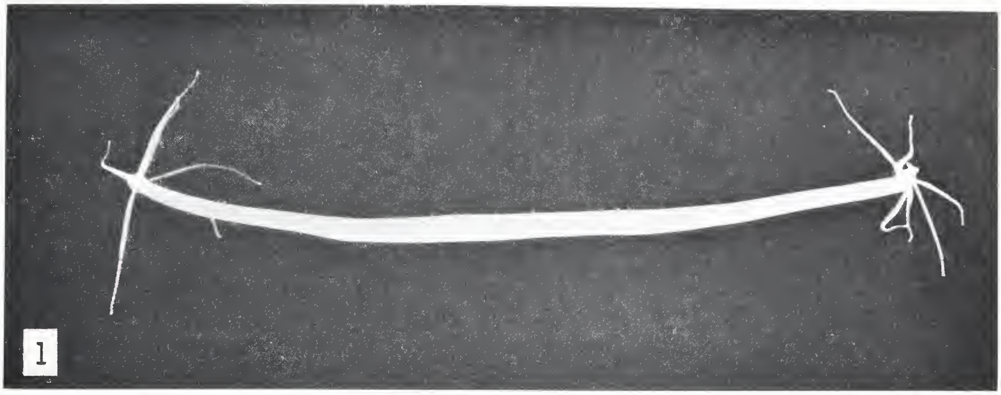
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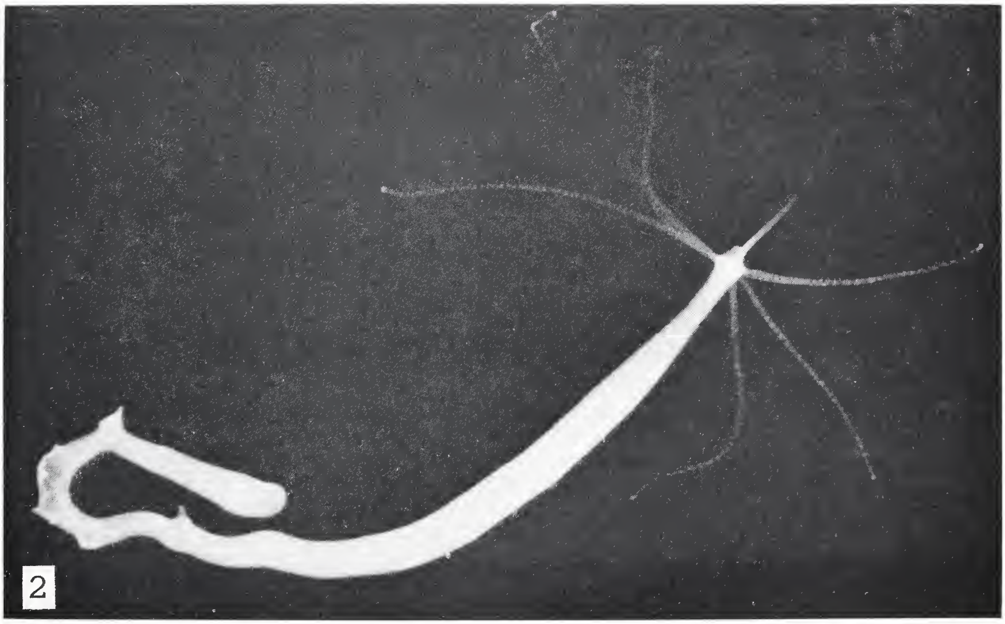
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FIGURE 1.—A bipolar mutant hydra derived from the apical portion of an animal that has "regenerated" a second head.

FIGURE 2.—Basal portion of a mutant hydra that has regenerated a head and is giving rise to small protrusions in the proximal region.

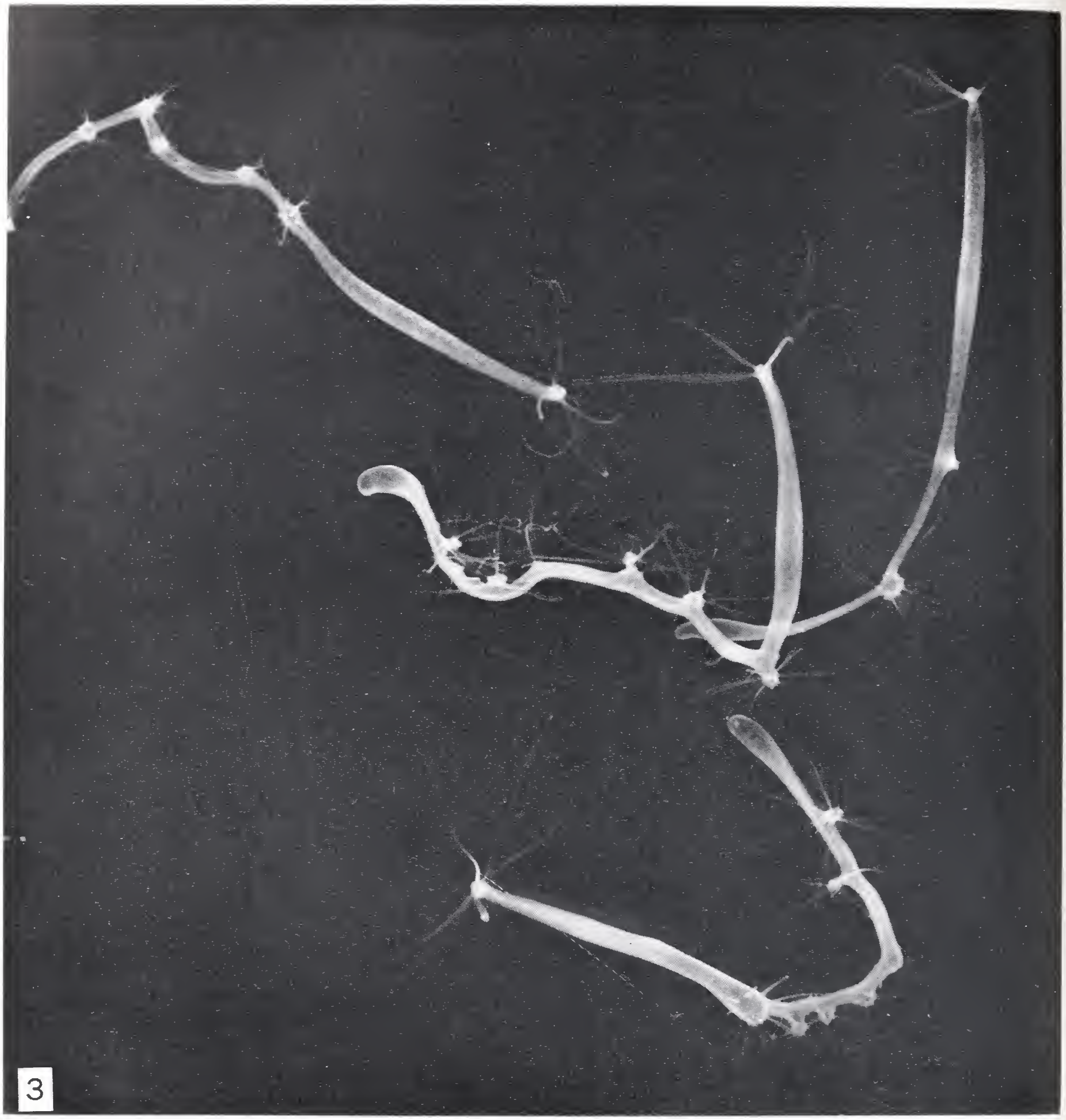


Figure 3.—Mutant hydra in which the proximal supernumerary tentacles form clusters.

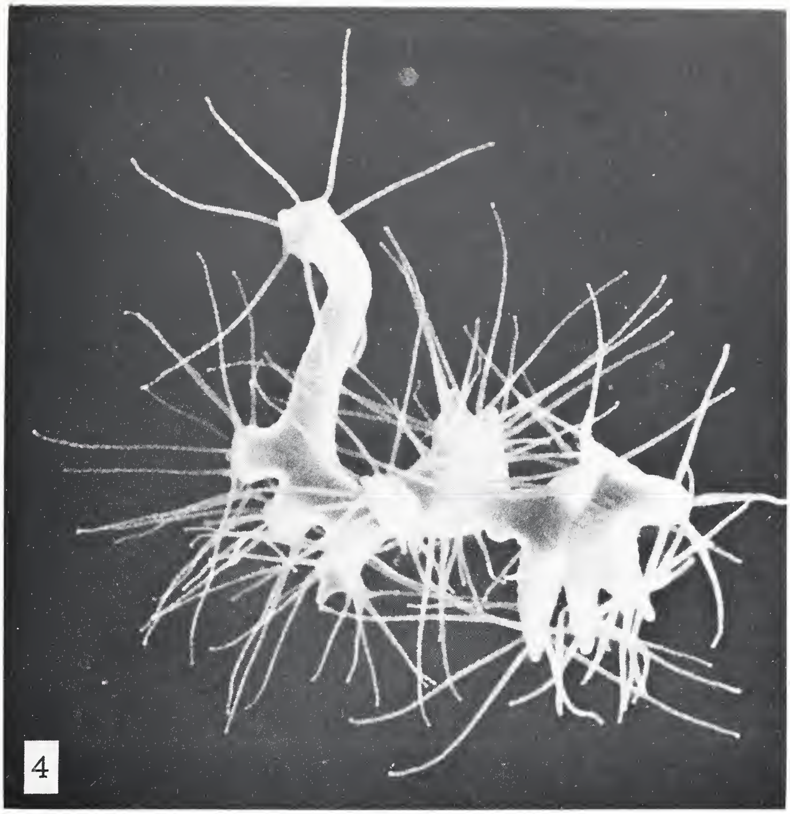
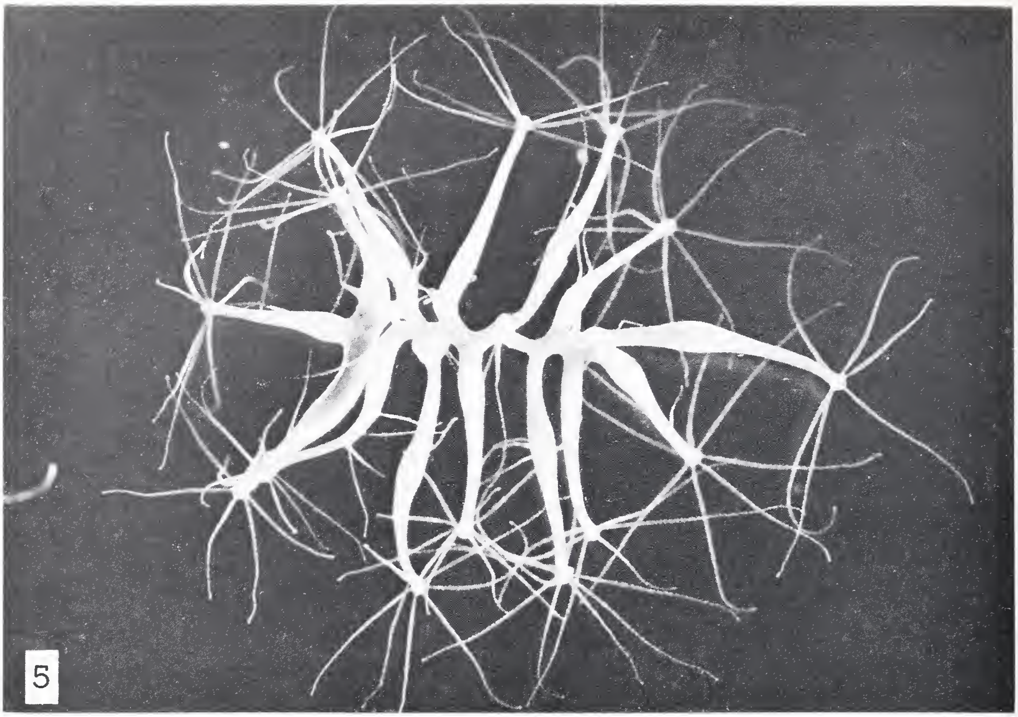


Figure 4.—Later stage of an animal from figure 3. *See* text.



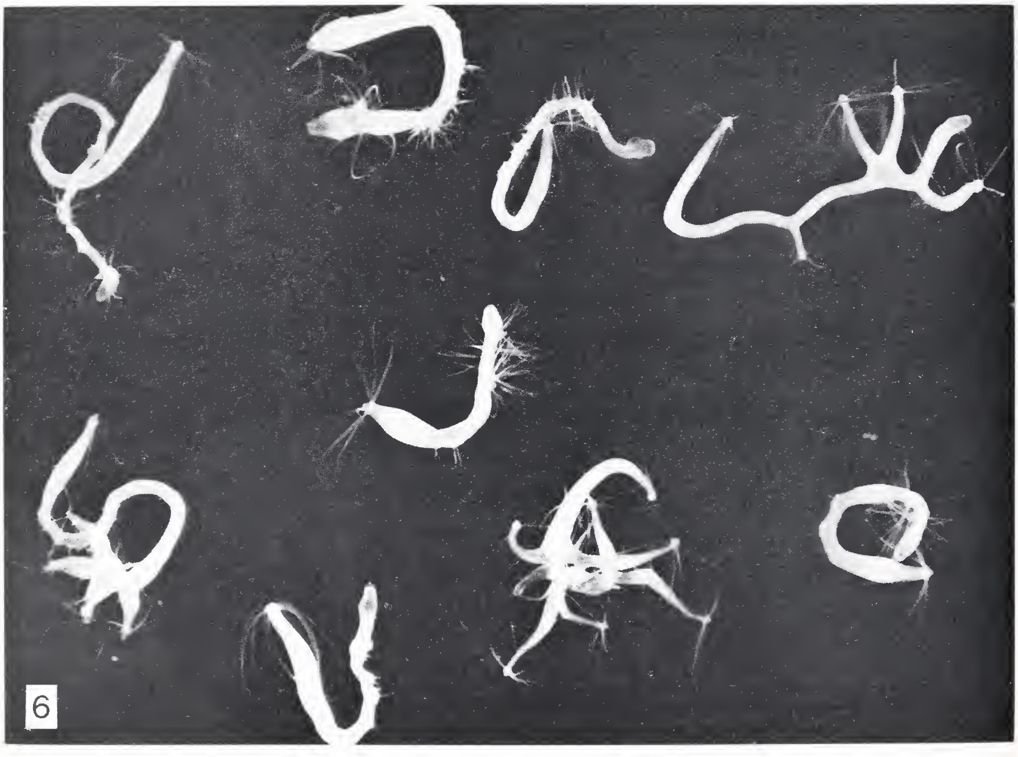


FIGURE 5.—A multipolar mutant hydra derived from the atypical regeneration of the basal portion of a mutant hydra that has gone through the stages illustrated in figures 2, 3, and 4.

FIGURE 6.—A clone of 9 mutant hydra derived from a single monopolar mutant by an atypical type of asexual reproduction over a period of 3 months.

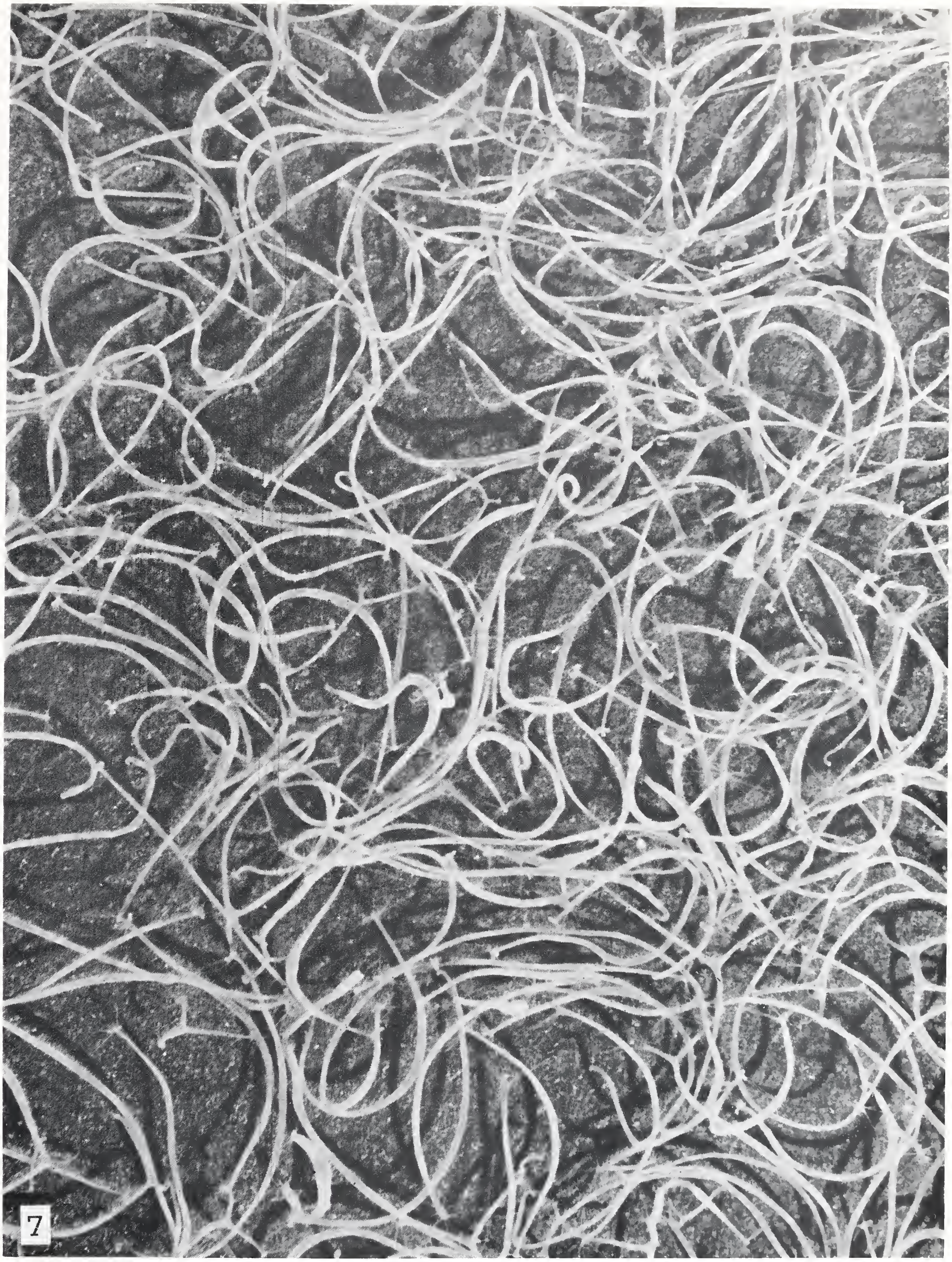


FIGURE 7.—Mutant-appearing heterocytes derived by budding of the normal-appearing heterocytes.

Supernumerary Heads in *Hydra viridis*

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SUMMARY

The mechanism of supernumerary head formation in regenerating *Hydra viridis* exposed to Colcemid was investigated. Optimal concentration and duration of exposure for promoting double-head formation were 0.1% Colcemid for 1 hour after amputating the distal parts of animals. Grafting amputated distal portions back to proximal portions of Colcemid-treated animals, or injecting Colcemid through the mouths of animals, also promoted the formation of secondary heads. Wounding and regeneration, therefore, were not prerequisite conditions for double-head formation. Nonbudding animals do not form 2 heads when treated, but animals engaged in bud formation do. Buds at the earliest stage of development (lacking tentacles and base) form secondary heads when present on animals at the time of treatment, while more advanced buds complete the development of their bases and detach. By grafting parts of green and white *H. viridis* together and following the movement of the graft border, it was established that cells move in a normal manner and at the same rates in each head. The retention of a bud as an additional head implies that the Colcemid treatment interferes with the formation of the bud's base. It appears not to act directly on the cells engaged in base formation, but on a stage of base formation preceding, by about 2 days, the visible appearance of the base.—Nat Cancer Inst Monogr 31: 739–750, 1969.

ABNORMAL GROWTHS in *Hydra* are not unknown. The formation of supernumerary heads or of ectopic tentacles is widely reported in the literature. Such growths may occur spontaneously (*1–3*) or as a result of several different treatments: exposure to the carcinogen dimethyl sulphate (*4*), to neurosecretory granules (*5*), to a material thought to act as a mitotic stimulator (*6, 7*), to lipoic acid (*8*), to Colcemid (*9*), or to puromycin (*9*), or by implanting distal parts of the body elsewhere (*10*).

In the present report, we analyze how *Hydra* develop supernumerary heads in response to Colcemid. Typically, double-headed animals were produced by placing normal single-headed ones in a Colcemid solution and sectioning them at a level midway between the ring of tentacles and the budding region.

MATERIALS AND METHODS

Animals were cultured, grafted, and photographed in the usual way (*11*). Injections were made by Campbell's technique (*12*). All experiments were performed on *Hydra viridis.* Crystalline Colcemid was obtained from Ciba Pharmaceutical Products Inc., Summit, New Jersey. Solutions were always made up just before being used since some activity was lost during storage.

RESULTS

The Double-Headed Condition

In figure 1, a double-headed animal (left) is compared to a normal single-headed one with an advanced bud protruding (right). Typically, one of the 2 heads (left) seems more nearly lateral and the other upright. A normal bud with tentacles as well developed as those on these heads would have begun to form its own base in anticipation of its detachment from the parent. There is, however, no evidence of base formation near the point of bifurcation in double-headed animals.

The point of attachment of the 2 heads may be at any level along the body. When only the ring of tentacles and the region just below are doubled, the level of the cleft may not change in the course of a week or more. Usually, the cleft forms at about the middle or in the lower half of the distance between the ring of tentacles and the budding region. Clefts in these areas move down into the budding region and, after several buds are formed, into the upper level of the peduncle. This may occur in about a week, but several weeks later the cleft may not have moved much farther. Ultimately, many of the originally double-headed animals split entirely into 2 single-headed animals, but others have remained double, attached through a common peduncle (fig. 2) for 2 months or more. Occasionally, the cleft from above is met by a cleft from below so that the last point of attachment is not at the very foot of the animal, but rather midway in the peduncle.

The production of supernumerary heads by treating decapitated animals with Colcemid need not stop at 2 heads. If the treatment is repeated, after an interval more heads are formed. Four days after the first treatment, the new heads are able to engage in feeding. The tentacles surrounding the 2 mouths capture prey and convey it into the appropriate mouth. There is no apparent cooperation between the sets of tentacles; indeed, there may be competition for the same larva. After resumption of feeding, there is growth of the heads and budding occurs. If, at this time, either or both heads are removed and the organism is again exposed to Colcemid, additional heads are likely to form. The animal shown in figure 3 has 4 heads.

Concentration

Several experiments were performed to determine optimum conditions for the formation of supernumerary heads. Groups of about 20 animals, chosen entirely at random, were taken from stock cultures 1 day after the last feeding. They were placed in dishes containing serial dilutions of between 10 μg/ml and 1 mg/ml Colcemid dissolved in culture medium. The animals were decapitated about half way between the ring of tentacles and the budding region and were exposed to Colcemid for 24 hours, after which they were returned to culture medium.

Normal wound healing occurs in the first few hours in the presence of Colcemid. After 24 hours, all the animals exposed to the highest concentration disintegrated, while animals exposed to lower concentrations began regeneration at a retarded pace.

After 2 days, head structures had appeared on almost all control animals (decapitated but not exposed to Colcemid). The hypostome was a conspicuous projection at the most distal level, and tentacles began as small knobs. In the 100 control animals observed during these experiments, only 1 formed a double head, and this by a conspicuously different route than that followed by the Colcemid-treated animals. In that control, an early bud present at the time of amputation usurped control of the remainder of the parent animal. No regeneration occurred at first. The wound marking the site of the original head did not seem to heal, however, and after a week another head grew at this site.

The regeneration of heads in Colcemid-treated animals was not as advanced after 2 days as in the controls. At this time, the animals that go on to form double heads may be bilobar at their distal end, but hypostomes and tentacles are only observed in half these animals. Only about 10% of these randomly selected animals form double heads after treatment with the lower concentrations of Colcemid. As many as 20% of the animals show some signs of degeneration, such as spewing digestive cells from the amputation surface. These animals ultimately disintegrate.

Exposure Time

Sixty animals were decapitated and placed in 0.1% Colcemid in culture medium. Samples of about 10 animals were removed periodically.

The rate of degeneration and disintegration after Colcemid treatment was a function of the duration of exposure. Within 1 day after treatment, all the animals exposed to Colcemid for about 8 hours had disintegrated. Those exposed for only 6 hours were degenerating; that is, they were balled-up and surrounded by loose clumps of cells. However, these animals did not disintegrate until the following day. Animals exposed to Colcemid for about 3 hours fared better, although regeneration was retarded as compared to those exposed to Colcemid for only 1 hour. Half of the latter had begun to form double heads by the 3d day after decapitation. The remaining animals formed single heads or degenerated with the same frequency.

Evidently, prolonged treatment is not necessary for the promotion of double-headedness. Indeed, the frequency of double-headedness is increased whereas mortality is not affected when high concentrations of Colcemid are used for short periods, in contrast to the results with lower concentrations used for longer periods.

Effects of Wounding and Regeneration

Normal animals put into a solution of Colcemid never formed double heads. The role of wounding in the process of double-head formation may be merely to expose the cells on the inner surface of the animal's body wall to Colcemid, or perhaps, something more complicated. Likewise, the process of regeneration may be a consequence of amputation and play no significant role in determining whether 2 heads are formed or, on the other hand, Colcemid may literally induce 2 heads to regenerate at the wound surface.

To determine whether regeneration played a necessary role, animals were bisected in the usual way in Colcemid (0.1%), but the pieces were grafted back together while still in the Colcemid. Eleven of twelve grafts healed together to reconstitute whole animals. Thus wounding was accomplished but regeneration did not accompany the process, the parent animal's head being restored by grafting. To determine whether sectioning played a necessary role, 20 animals were given injections of 1.0 μl of 0.1% Colcemid by insertion of a glass needle into their mouths. These animals were kept in 0.1% Colcemid for 1 hour and then returned to culture medium. Thus the cells on the inner surface of the animal's body wall as well as those on the outer surface had been exposed to Colcemid even though the animal was not sectioned. The animals in both experiments were rapidly budding adults.

Five of the grafted animals and 4 of the animals receiving injections subsequently formed supernumerary heads. These heads formed at the budding region in most cases. One grafted animal formed 3 additional heads, all in the budding region. Ten animals given injections of culture medium failed to develop supernumerary heads. We have never produced supernumerary heads by grafting in culture medium. Neither wounding nor regeneration, therefore, is necessary for the production of double heads if the inner surface of the animal's body wall can be exposed to Colcemid.

Role of Budding

In an effort to understand why all the animals exposed to Colcemid did not respond uniformly, we considered the possibility that the developmental stage of the animal may play a role. The effects of amputation and Colcemid (0.1% for 1 hour) on freshly detached animals (those detaching from parents within 24 hours) were compared to the effects on rapidly budding parents. Only 1 double-headed animal of 20 was found among the treated, freshly detached animals, whereas most of the treated, rapidly budding animals formed double heads.

Since freshly detached animals do not generally have buds developing on them, it seemed that budding may have played a role. Groups of about 10 animals with no buds, 1 bud, or 2 buds and a group of about 20 animals with 3 buds were amputated and placed in the Colcemid solution (0.1%) for 1 hour to determine if the degree of budding was correlated with the frequency of double-head formation. None of the animals lacking buds developed 2 heads. Only 1 of those having 1 bud at decapitation developed a double head, whereas half of those with 2 buds developed double heads and 15 of those with 3 buds developed double heads.

This correlation raised the possibility that a secondary head may form on a parent by the retention of a bud that would normally have detached. To test this possibility, 6 animals with 2–4 buds were photographed before treatment, after the amputation of their heads, and on consecutive days thereafter. The fate of each bud was strictly observed.

Four of the animals went on to form double heads. Like the one shown in figures 4–9, each of these animals had a bud in a very early stage at the time of treatment (*arrow*, fig. 4). Indeed, it appeared in each case that these early buds developed into lateral secondary heads (arrows, figs. 5–7). All of the more advanced buds formed their own bases within 1–3 days and detached from the parents in the normal fashion (figs. 7 and 8).

To test the possibility that buds in an older stage could also form secondary heads had the buds in the earlier stage not been present, 60 animals, each having but one bud, were treated. These buds were classified as to whether tentacles were present or not and whether a base had begun to form. Upon regeneration, 10 animals had formed double heads. All of these had had buds at the earliest stage of development bearing no tentacles and no bases. These buds were at the stage illustrated at the arrow in figure 4. None of the animals with buds bearing conspicuous tentacles at the time of treatment formed double heads, with the exception of 1 animal that had formed a new bud 1 day after treatment. This bud formed the secondary head. All of the more advanced buds went on to form or complete their bases and subsequently to detach. Younger buds did not, therefore, inhibit the reaction of the older buds; rather, only the former remained attached to the parent in response to the treatment.

Cell Movements in Supernumerary Heads

The final question was whether the 2 heads of double-headed animals were equivalent and whether both had normal tissue dynamics. In agreement with Turner (*3*), who observed spontaneously occurring double-headed animals, we observed that the budding rates of animals were a function of the number of budding regions present: When only 1 budding region was present, double-headed animals budded at the normal rate, having about 2 buds attached at any one time; subsequently, as a result of continued fission, when 2 budding regions were present, 2 or more buds were present in each region so that the rate of budding for the animal as a whole had doubled. Since, normally, parental body cells move down the length of the gastric region into the budding region and out onto buds (*11, 12*), the equivalence of the heads and the degree of their normalcy would be indicated by the downward movement of cells on both heads.

Animals of the species *H. viridis* can be raised either to contain or to lack a green algal symbiont within the cells lining the inner surface of their body wall. The algae impart a green color to the animal. Animals lacking the algae are white. By grafting green and white animals together, a border is formed which can be observed over the course of several days. The movement of this border corresponds to the movement of the hydra's inner surface cells. Our experiences with grafts of these types, in which green heads are placed on white bottoms or vice versa, fail to indicate any infiltration of algae into cells originally lacking algae, at least for several days after grafting.

In 5 grafts, the green-white borders were placed about midway between the tentacles and the budding regions. In these animals, the cleft between the 2 heads had extended to, or into, the budding region (fig. 10). One day later (fig. 11), the borders appeared relatively lower in the animal, growth having occurred above them. On the following day, the border had moved into the budding region (fig. 12) and buds began to appear with the color of the originally distal piece. This downward movement of the border seemed to occur at about the same rate in the 2 heads. It should be noted here that, while the cleft was also presumably moving down, the border marking the position of cells in the inner surface of the body wall moved down faster.

Cells in the most distal region of a normal animal are known to move out onto tentacles. One white animal, in which only the rings of tentacles and subjacent parts of the body wall were doubled, was also used in grafting. The head structures were replaced by comparable pieces of green animals, and the green-white borders were observed. After several days, the borders had moved out onto the tentacles, just as they would have moved in one-headed animals. Again, the borders moved at about the same rates. However, the cleft between the 2 heads did not move conspicuously during this period.

These observations indicate, therefore, that the heads are equivalent and that the cells in the inner layer of double-headed animals move as they do in normal animals. In addition, the level of the cleft between the heads and its movement obviously do not depend on the dynamics of these cells, since the cells and the cleft can move at different rates.

DISCUSSION

It would appear that the term "abnormal growth" would be incorrectly applied to the double-headed animals studied here: Secondary heads arise from normal buds and the two-headed animals have the same growth dynamics as normal animals. While the presence of 2 or more heads is, of course, abnormal for *Hydra*, these heads are neither produced by abnormal growth nor do they grow abnormally. What is abnormal is the failure of the original bud to form its base and detach. Colcemid somehow interferes with the differentiation of the bud's base. The bud does not reach its normal endpoint of development on the parent.

It would seem that the Colcemid does not act directly on cells engaged in base differentiation or on those soon to do so: Buds in the advanced stages formed their bases and detached despite the treatment. Even buds that had small tentacles and no gross indication of developing bases at the time of treatment went on to complete their bases and detach 2 or more days later.

The effect of Colcemid seems to be on a stage of, or process necessary for, base formation that precedes the visible appearance of the base by some 2–3 days: Only buds at the earliest stages of development, at the time their parents were treated, subsequently failed to form their bases.

One might have hypothesized that there are direct effects of the Colcemid on differentiating base cells, but these effects are latent in the early buds and only materialize when the base is forming. It would be difficult, however, to explain with such an hypothesis how bases formed on the older buds that were soon to be or were already engaged in base formation at the time of treatment.

The failure of the secondary heads to form bases is surprising, since *Hydra* is famous for its ability to regenerate missing parts. Why, we may ask, does the affected bud fail to regenerate a base even if it does not differentiate in the normal sequence? A mechanism which might trigger regeneration by telling an animal that it does not have the appropriate form may have failed to work after Colcemid treatment, or this mechanism may be incapable of distinguishing between one and two normally growing heads. Another possibility is that the mechanism operates, but the susceptible cells are not available, or that other appropriate conditions are not realized.

The literature on *Hydra* is one of the forums in which the question of morphogenetically significant gradients is debated. Indeed, Webster's purpose in investigating the effects of Colcemid (*9*) was to study what he calls "time-threshold factors." For instance, one such factor might originate in the head and inhibit the formation of other heads. If only animals whose heads had been removed had responded to Colcemid by forming 2 heads, there would be some reason to speculate on the importance of such factors here. However, amputation of the parent's head was not necessary for the production of double-headed animals. It would seem, therefore, that either the head of the animal is not a necessary source of a gradient involved in inhibiting head formation, or that such a gradient does not exist.

These results have established that even the grossly abnormal double-headed hydras grow exquisitely normally. The formation of supernumerary heads is not due to the induction of abnormal growths but to the prevention of a normal endpoint in development. The ultimate regulation of double-headed animals into 2 single-headed animals suggests that the "cure" for "abnormal growth" of this type is not to be found in inhibiting their growth, but rather in promoting their development so that they reach their normal endpoints.

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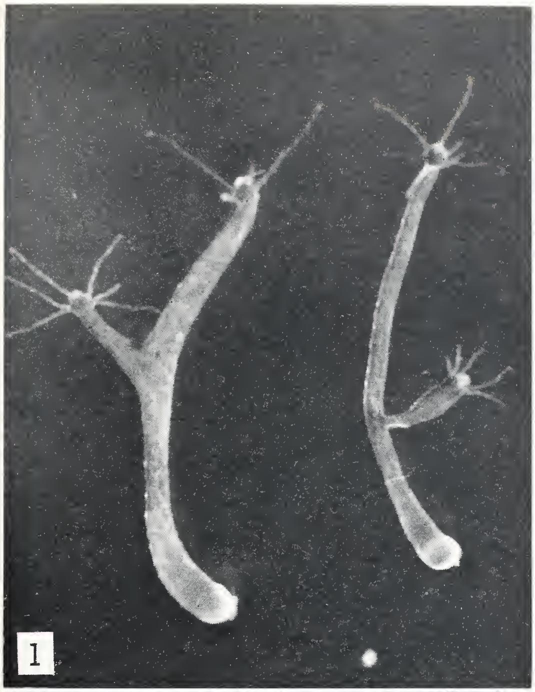
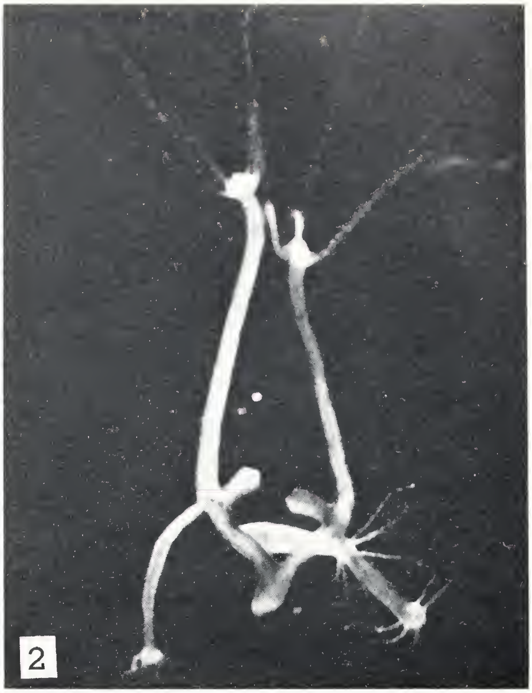
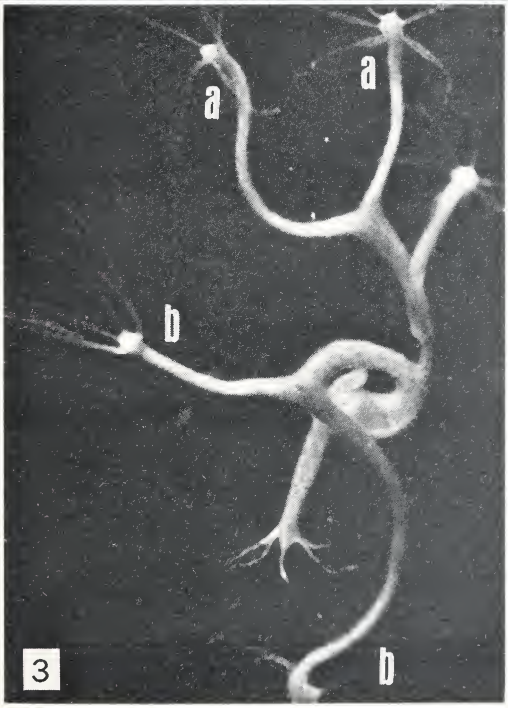




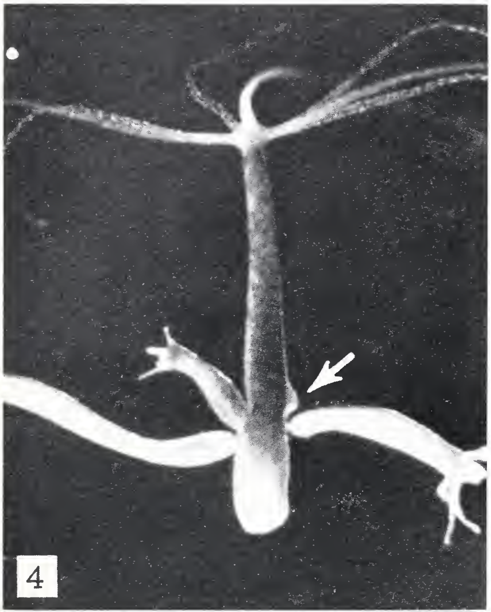


Figures 1–3.—Compare normal, double animals at different stages of fission, and quadruple animals. × 13

Figure 1.—Double-headed animal (*left*) and normal animal with advanced bud (*right*). Secondary head is more broadly attached to remainder of parent animal than the bud which has begun to form a base.

Figure 2.—Double animal in which cleft between nearly symmetrical parts has penetrated deeply into peduncle. There are two budding regions bearing 2 and 3 buds.

Figure 3.—Four-headed animal (*a*, *a*, *b*, *b*) with 2 budding regions, each bearing an advanced bud.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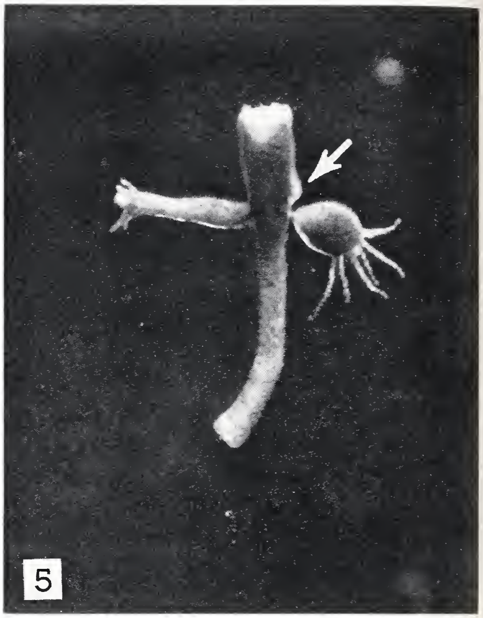
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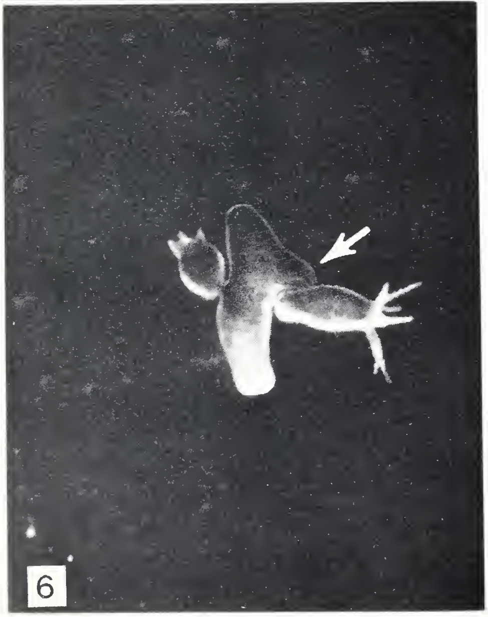
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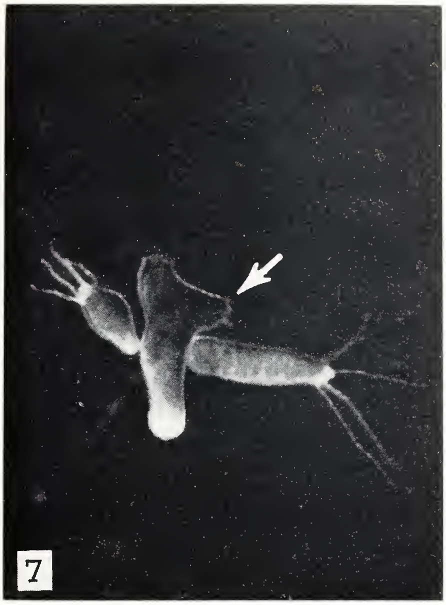
Figures 4–9.—The formation of a secondary head from a young bud. Same animal with its orientation preserved is shown before, during, and after Colcemid treatment. × 13

Figure 4.—Bud at beginning of its development (*arrow*) and 3 advanced buds are present in budding region of parent animal.

Figure 5.—Fifteen minutes after amputating distal part of animal. One of the older buds detached during handling. Advanced bud on *right* is contracted. Younger bud (*arrow*) is unchanged. Peduncle (region below budding region) is expanded.

Figure 6.—One day after treatment. Distal end appears bilobar. Early bud (*arrow*) gave rise to lateral lobe which resembles healed medial lobe.



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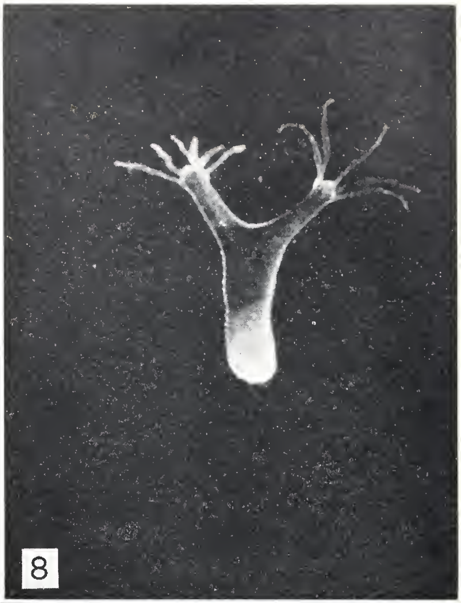

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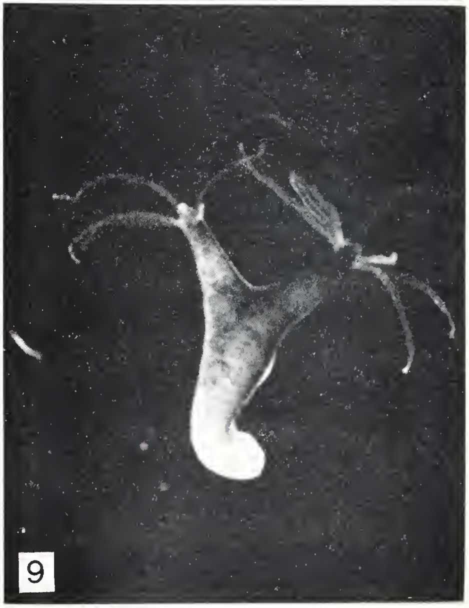

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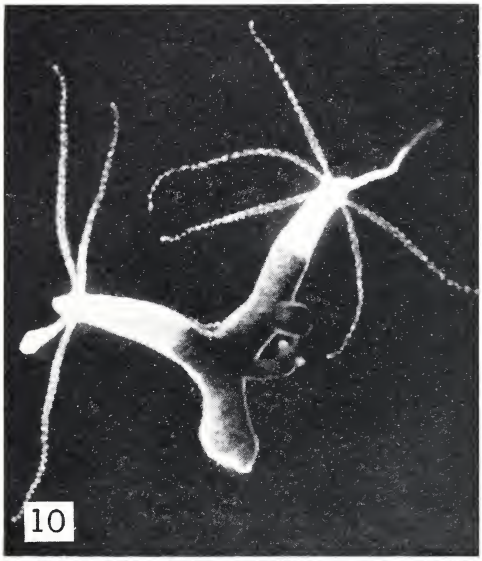
FIGURE 7.—Two days after treatment. Small developing tentacles are present on protuberance formed by the bud (*arrow*). Smaller knobs have formed at site of regeneration.

FIGURE 8.—Six days after treatment. Regeneration of distal parts and retention and growth of bud have resulted in formation of a double-headed animal. The two advanced buds completed their development and detached in the intervening time.

FIGURE 9.—Seven days after treatment. Heads continue to eat and grow. Two heads now appear equivalent. Neither head shows any sign of forming a base.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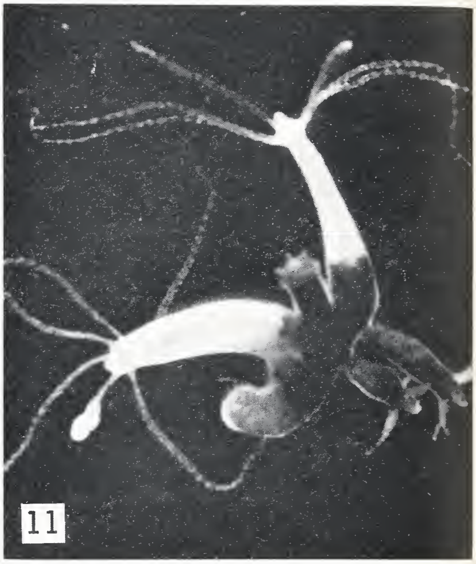
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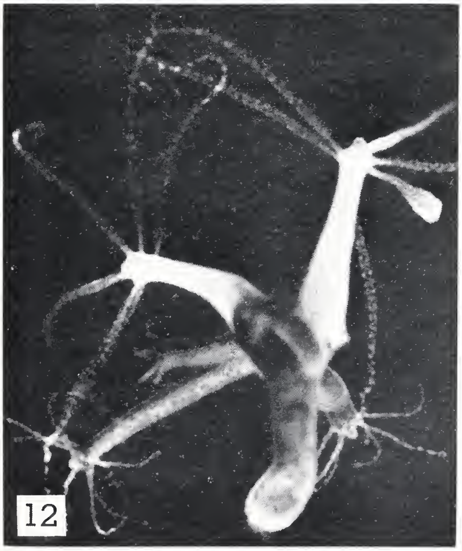
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Figures 10–12.—Downward movements of borders formed by grafting white distal parts to a green proximal part of a double animal. × 13

Figure 10.—One day after grafting. Two relatively early buds are present.

Figure 11.—The following day. Graft borders have moved closer to point of bifurcation while, at the same time, distal growth places borders further from hypostomes. Two more buds have begun and the two present in figure 10 have advanced in their development.

Figure 12.—The third day. Graft border in right-hand member has moved into budding region. A rudimentary white bud is seen emerging on *right*. One advanced green bud obscures border in left-hand member, but this border has also moved closer to point of bifurcation.

Neoplastic Equivalents of Protozoa[1]

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SUMMARY

Biochemical studies on the cytoplasmic particle λ of *Paramecium aurelia* are reviewed. These studies have shown that this particle is an endosymbiote; this probably holds true for other related particles such as K, μ, and π. The status of the "metagon," once thought to be messenger RNA necessary for μ production, is in doubt. Failure to repeat the original observations is probably due to the cultivation of paramecia in bacterially contaminated cultures. An analogy can be drawn between cytoplasmic λ-particles and plastids and mitochondria. The λ-particles might have evolved from infectious agents into cytoplasmic components conferring ecological advantages, and, as such, can be looked upon as "neoplastic equivalents."—Nat Cancer Inst Monogr 31: 751–769, 1969.

WEBSTER'S *Third New International Dictionary* (Unabridged) defines a neoplasm as follows: "neoplasm: a new growth of animal or plant tissue, resembling more or less the tissue from which it arises but serving no physiologic function and being benign, potentially malignant, or malignant in character." Two of the definitions of an equivalent are: 1) that of being a counterpart or 2) one that is virtually identical in value, meaning, or effect.

Can we on this basis designate certain features of protozoa as neoplastic equivalents? I believe we can, as long as we are not as arbitrary as Humpty Dumpty when he replied to Alice: "When I use a word, it means just what I choose it to mean, neither more, nor less."

Cytoplasmic particles have been reported to be present in several ciliates. Chatton and Lwoff (*1*) found, in 1929, that the ciliate *Ellobiophrya donacis* contains in its cytoplasm rods from 8–20 μ long, some of which appeared to be dividing. Because of the appearance of these rods, their presumed division, and their presence in all examined specimens, indicating transmission to the offspring during reproduction, the particles were believed

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[2] I wish to express my appreciation to Mrs. Betty Stauderman for her patience and expertise in typing this manuscript and to Mr. Robert Rietberg for his excellent drawings.

to be symbiotic bacteria. Fauré-Fremiet (*2*) observed Feulgen-positive particles in the cytoplasm of all specimens in collections of the ciliates *Euplotes patella* and *E. eurystomus*. The particles were gram-negative filaments, 5–12 μ long. They were very sensitive to penicillin, but not to aureomycin, streptomycin, chloromycetin, or the sulfonamides.

In 1957, Newton and Horne (*3*) described basophilic, rod-shaped particles in the cytoplasm of the trypanosomid flagellate, *Crithidia* (*Strigomonas*) *oncopelti*. The particles were composed largely of ribonucleoprotein, but no conclusion was reached in regard to their nature.

The existence of the cytoplasmic particles κ in *Paramecium aurelia*, stock 51, Syngen 2, its cytoplasmic localization, and its reproduction were at first inferred from indirect evidence. Sonneborn (*4–6*) found that certain strains of paramecium liberated an agent into the culture medium that killed paramecia of other strains. He postulated that the production and liberation of the killing agent depended on the presence in the killer paramecium of a cytoplasmic factor "κ." Preer (*7*, *8*) was able to render the κ-particle visible by using the Feulgen and the acid-Giemsa stains.

With the killer trait as an indicator (spinning, humping, paralysis, vacuolization, rapid lysis, and mate-killing), the existence of other cytoplasmic factors was recognized (*9*). These particles, such as λ (rapid lysis), μ (mate-killing), π (a nonkilling mutant of κ), were subsequently visualized.

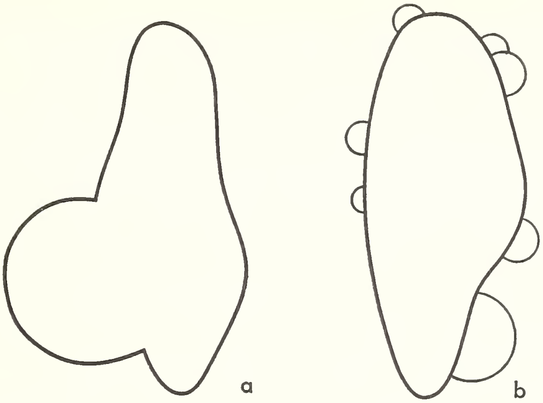
Soldo (*10*) found that the presence of particles in the cytoplasm of *P. aurelia* and other ciliates could be easily demonstrated by using the aceto-orcein stain (figs. 1–4).

Of the particles present in *P. aurelia*, the κ-, λ-, and μ-particles have been investigated most thoroughly with regard to their development, morphology, cytochemistry, reproduction, transmission, maintenance in the host, and their biochemistry.

P. aurelia, stock 51, containing the κ-particle in their cytoplasm, liberate killing particles into the medium that cause sensitive paramecia to develop a characteristic aboral hump before they die (*5*, *6*) (text-fig. 1a). *P. aurelia*, stock 299, containing λ-particles (*11*), cause sensitive animals to develop many clear pellicular blisters, resulting in death by lysis within 20 minutes (text-fig. 1b). The "mate-killers" (*12–15*), containing the μ-particle, kill sensitives only by prolonged direct contact such as occurs during mating. The sensitive mate does not die until after conjugation.

These particles multiply in the cytoplasm of the killer paramecia. Approximately half of the total number of particles is transmitted to each daughter cell at binary fission. The full population level is restored before the next fission. The particle-bearing paramecia are resistant to the killing action of their own particles.

The genotype of the paramecia is decisive for one feature of the killer system. It determines whether the particles will be maintained in the cytoplasm or whether they will disappear. *Kappa*-51 will be maintained only when the paramecia contain the gene *K*, either *KK* or *Kk*. *Kappa* is not maintained in *kk* cells. When *Kk* paramecia containing κ conjugate



TEXT-FIGURE 1.—Schematic drawing of κ-killing (a) and λ-killing (b) particles.

with either *Kk* or *kk* paramecia, those that acquire the *kk* genotype produce clones in which κ has irreversibly disappeared. The same holds for the μ-particle. Populations of μ are maintained only in clones possessing the gene *M;* in *mm* clones, μ disappears. The maintenance of λ is also under genetic control of a gene *L* (*16*).

BIOCHEMISTRY OF THE κ- AND λ-PARTICLES

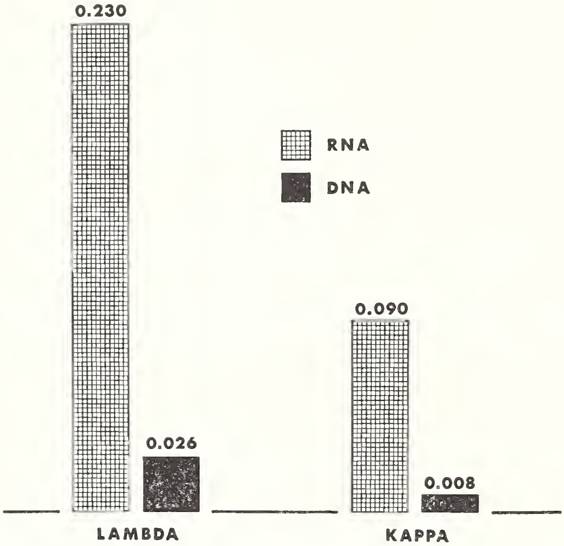
The biochemical investigations on the λ-particle have become feasible with the advent of an axenic medium for the growth of the λ-bearing *P. aurelia* (*17*). Although we have not yet been able to grow κ-bearing paramecia in axenic culture, Dr. Joan Smith-Sonneborn was able, by using elaborate controls, to carry out several chemical determinations on κ.

Preer (*7, 8*) had established that the κ-particle was Feulgen-positive and stained heavily with acid-Giemsa. From this it was inferred that the κ-particle contained relatively large amounts of DNA.

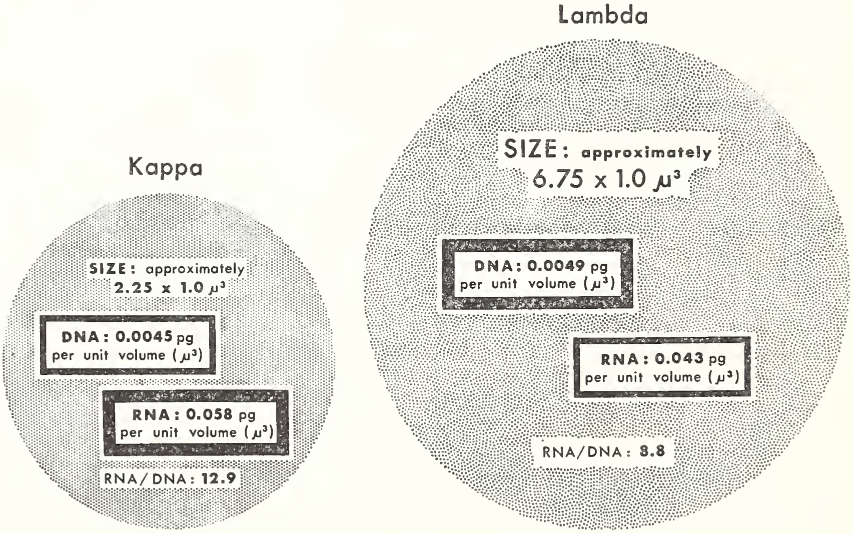
When we started to investigate the λ-particle, we found it stained with aceto-orcein, but was Feulgen-negative. Furthermore, the λ-particle stained positive with Pyronin Y-Methyl Green, indicating the presence of RNA. After treatment of the λ-bearers with ribonuclease, no Pyronin Y-Methyl Green-positive rods could be found in the cytoplasm (figs. 5–7).

Thus we originally believed that the λ-particles were fundamentally different from the κ-particles, in that the former were composed of RNA and the latter of DNA. We were very much surprised when a chemical analysis of the isolated and purified κ-particles by Smith-Sonneborn (*18*) and of the λ-particles by Tanguay (*19*) revealed that a single κ-particle contains 8×10^{-3} pg of DNA and 9×10^{-2} pg of RNA. A single λ-particle, on the other hand, contains 26×10^{-3} pg of DNA and 23×10^{-2} pg of RNA (text-fig. 2). Actually the λ-particle contains more DNA than the κ-particle. The λ-particle is larger than the κ-particle and this, of course, could explain why more DNA was found. These data become more striking when one expresses them on a unit volume basis (text-fig. 3).

On a unit volume basis (μ^3) the κ-particle contains 45×10^{-4} pg of DNA and 58×10^{-3} pg of RNA, as compared to the λ-particle, which contains on this basis 49×10^{-4} pg of DNA and 43×10^{-3} pg of RNA. The RNA/DNA ratio for the κ-particle is 12.9 and for the λ-particle 8.8. It is thus surprising that the λ-particle does not stain with the Feulgen procedure.



TEXT FIGURE 2.—RNA and DNA content of the λ- and the κ-particles.


Lambda
SIZE: approximately 6.75 x 1.0 μ^3
DNA: 0.0049 pg per unit volume (μ^3)
RNA: 0.043 pg per unit volume (μ^3)
RNA/DNA: 8.8
Kappa
SIZE: approximately 2.25 x 1.0 μ^3
DNA: 0.0045 pg per unit volume (μ^3)
RNA: 0.058 pg per unit volume (μ^3)
RNA/DNA: 12.9

TEXT-FIGURE 3.—A comparison of the κ- and the λ-particles.

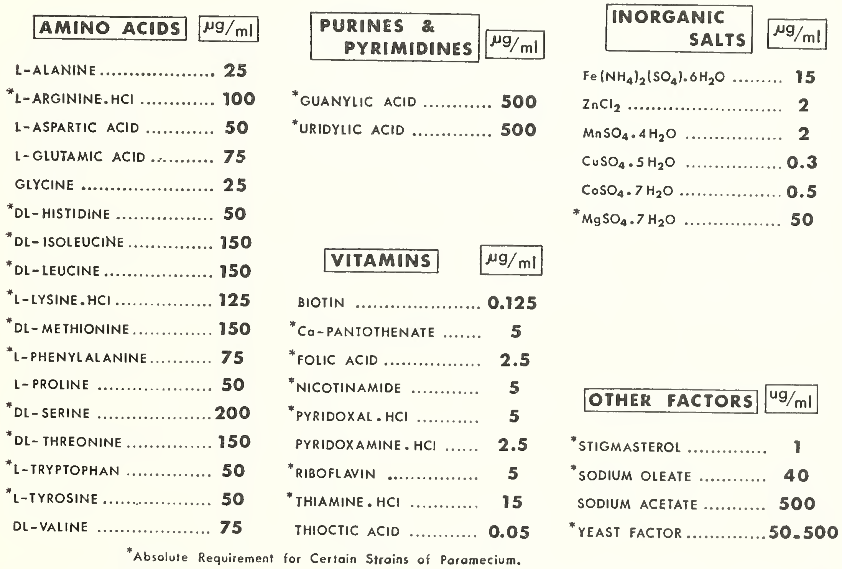
Soldo, Reid, and van Wagtendonk (*20*) found that the DNA extracted from purified λ-particles contains one component which exhibits the properties of a single-stranded DNA.

To establish the biological status of λ, Soldo (*21*) further investigated the λ-bearers, and their λ-less counterparts. These organisms were selected because both could be grown successfully in great quantities in an axenic medium, in which the only unknown constituent was a nondialyzable, nucleotide-free fraction obtained from a water-soluble extract from bakers' yeast. The medium consists of 17 amino acids, 9 vitamins, guanylic acid, uridylic acid, 6 organic salts, and stigmasterol, sodium oleate, sodium acetate, and the yeast factor. With this medium it was possible to screen for some of the contributions that the λ-particles might make to the nutritional requirements of the host, especially with regard to the nucleotide and vitamin requirements (text-fig. 4).

Text-figure 5 shows that the λ-bearing and the λ-free paramecia grow equally well when either guanosine or guanylic acid was added to the medium, but neither strain grew when they were omitted from the medium.

The 299 λ and the 299 λ-less organisms grew equally well when either cytidine, uridine, 5′-cytidylic acid, or 5′-uridylic acid was added to the medium (text-fig. 6).

In the absence of any of the pyrimidine derivatives, neither strain was able to grow in the medium. It can thus be concluded that the λ-particles do not contribute the essential purines or pyrimides to the nutritional requirements of the host.



AMINO ACIDS	µg/ml
L-ALANINE	25
*L-ARGININE.HCl	100
L-ASPARTIC ACID	50
L-GLUTAMIC ACID	75
GLYCINE	25
*DL-HISTIDINE	50
*DL-ISOLEUCINE	150
*DL-LEUCINE	150
*L-LYSINE.HCl	125
*DL-METHIONINE	150
*L-PHENYLALANINE	75
L-PROLINE	50
*DL-SERINE	200
*DL-THREONINE	150
*L-TRYPTOPHAN	50
*L-TYROSINE	50
DL-VALINE	75

PURINES & PYRIMIDINES	µg/ml
*GUANYLIC ACID	500
*URIDYLIC ACID	500

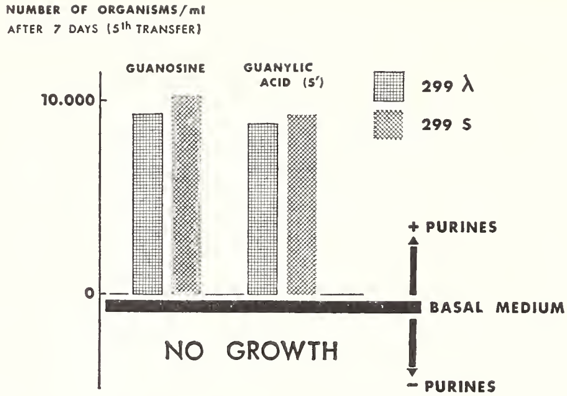
VITAMINS	µg/ml
BIOTIN	0.125
*Ca-PANTOTHENATE	5
*FOLIC ACID	2.5
*NICOTINAMIDE	5
*PYRIDOXAL.HCl	5
PYRIDOXAMINE.HCl	2.5
*RIBOFLAVIN	5
*THIAMINE.HCl	15
THIOCTIC ACID	0.05

INORGANIC SALTS	µg/ml
$Fe(NH_4)_2(SO_4).6H_2O$	15
$ZnCl_2$	2
$MnSO_4.4H_2O$	2
$CuSO_4.5H_2O$	0.3
$CoSO_4.7H_2O$	0.5
*$MgSO_4.7H_2O$	50

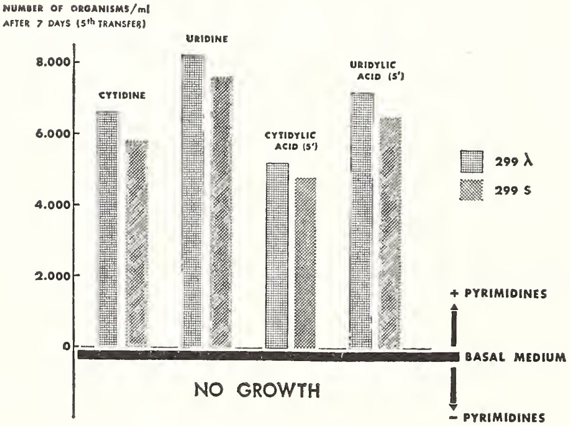
OTHER FACTORS	ug/ml
*STIGMASTEROL	1
*SODIUM OLEATE	40
SODIUM ACETATE	500
*YEAST FACTOR	50-500

*Absolute Requirement for Certain Strains of Paramecium.

TEXT-FIGURE 4.—Composition of the medium for theic axenic culture of λ-bearing and λ-free paramecium.



Text-figure 5.—Effect of omission of the essential purines on the growth of *P. aurelia,* stock 299 λ and 299 S.



Text-figure 6.—Effect of omission of the essential pyrimidines on the growth of *P. aurelia,* stock 299 λ and 299 S.

An analysis of the vitamin requirements of both strains revealed that, under the conditions of the experiments, neither strain appeared to require biotin or thioctic acid. A small amount of growth occurred in the absence of pantothenic acid. This could be due to a limited capacity of the organism to synthesize pantothenic acid or to the presence of suboptimal amounts of pantothenic acid in the yeast extract (text-fig. 7).

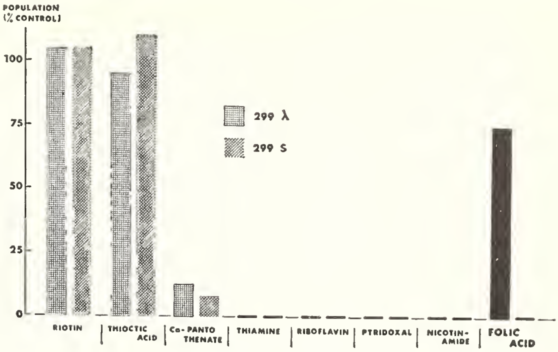
Often no growth was obtained in the first transfer, when thiamine, riboflavin, pyridoxal, or nicotinamide were omitted from the medium. Neither the λ-bearers nor the λ-less paramecia would grow in the absence of these vitamins.

It can be concluded from these experiments that the λ-particles do not contribute thiamine, riboflavin, pyridoxal, or nicotinamide to the host.

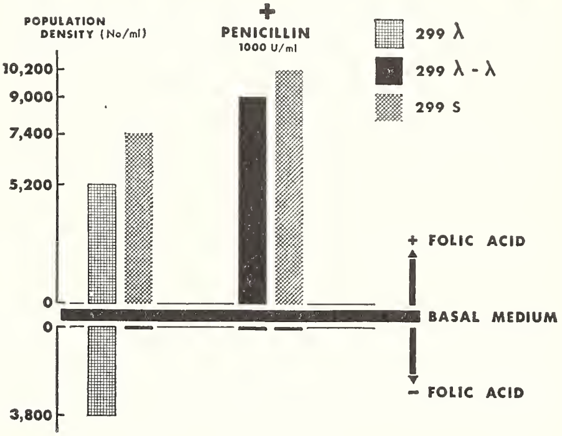
The growth of the λ-bearers in a folic acid-deficient medium, while the λ-less paramecia failed to grow in such a medium, indicated that the λ-particles contributed to the organism either folic acid or one of its precursors that *P. aurelia* was unable to synthesize.

The following experiments affirmed this hypothesis (text-fig. 8).

In the control experiment the λ-bearers and the λ-free organisms grew equally well in a medium containing folic acid. When folic acid was



Text-figure 7.—Effect of omission of vitamins on the growth of *P. aurelia*, stock 299 λ and 299 S.



Text-figure 8.—Effect of penicillin on the folic acid requirements of *P. aurelia*, stock 299 λ and 299 S.

omitted from the medium, only the λ-bearers were able to grow. When λ-bearing and λ-free organisms were grown in the presence of penicillin (which destroys the λ-particles and does not affect the growth of *P. aurelia*), both strains continued to grow in a folic acid-containing medium. However, when penicillin was added to λ-bearers growing in a folic acid-deficient medium, growth ceased. After the destruction of the λ-particles, the λ-free organisms could no longer maintain themselves without folic acid.

Finally, van Wagtendonk and coworkers (*22*) were able to grow the λ-particles *in vitro* in the following medium (text-fig. 9). Growth in this medium is far from luxurious, approximately one fission every 2 days.

A series of studies comparing the respiration of the κ-bearing stock 51 with that of three κ-less stocks was carried out by van Wagtendonk and his associates (*23–26*). The respiratory rate of the killer cells was almost double that of the sensitives. The higher Q_{O_2} of the killers as compared with the sensitives, the lack of respiratory inhibition by sodium azide in concentrations inhibiting the respiration of the sensitives, and the low cytochrome oxidase activity of the killers support the conclusion that an oxidative system different from the normal cytochrome system is operating in the killers. A succinoxidase system is associated with the presence of κ.

AMINO ACIDS	µg/ml
L-ALANINE	60
L-ARGININE HCl	60
L-ASPARTIC ACID	20
DL-ASPARAGINE	36
L-GLUTAMIC ACID	80
GLYCINE	40
DL-HISTIDINE HCl	40
DL-ISOLEUCINE	50
DL-LEUCINE	50
L-LYSINE HCl	50
DL-METHIONINE	60
L-PHENYLALANINE	40
L-PROLINE	40
DL-SERINE	60
DL-THREONINE	70
L-TRYPTOPHAN	30
L-TYROSINE	15
DL-VALINE	40

PURINES & PYRIMIDINES	µg/ml
ADENOSINE	500
GUANOSINE-MONO-PHOSPHATE	250
URIDINE-MONO-PHOSPHATE	250

SALTS	µg/ml
$MgSO_4 \cdot 7H_2O$	250 ⊛
0.001M Na-PHOSPHATE pH 6.8	20
Na EDTA	500

VITAMINS	µg/ml
BIOTIN	1.25 ⊙
Ca PANTOTHENATE	5
FOLIC ACID	2.5
NICOTINAMIDE	5
PYRIDOXAL HCl	5
PYRIDOXAMINE HCl	2.5
RIBOFLAVIN	5
THIAMINE HCl	15
THIOCTIC ACID	0.05

OTHER FACTORS	µg/ml
EDAMINE S	5 *
GLYCEROL	10 *
MALTOSE	2.5 *
STIGMASTEROL	0.4
YEAST FRACTION	500

⊛ ml/100ml ⊙ mµg * mg

TEXT-FIGURE 9.—Composition of the medium for the growth of the λ-particles.

Simonsen and van Wagtendonk (*23*) suggested that the respiratory system associated with the presence of κ might be a flavin system. The reduced cytochrome oxidase activity of killer homogenates indicated that the number of mitochondria was reduced in the presence of κ.

κ, μ, AND THE METAGON

The metagon hypothesis had its origin in the initial observations by Chao (*27*). He claimed that κ persisted in all observed descendants of cells which had become homozygous recessive at conjugation (*Kk*→*kk*) for 7 cell generations. In the 8th cell generation, κ suddenly disappeared completely from a small number of the cells. In the 15th cell generation no cells containing κ could be found. During this period those cells which continued to contain κ had amounts in the normal range for heterozygotes. Apparently, κ multiplied as usual or disappeared suddenly.

Gibson and Beale (*28*) observed that μ 540 apparently was lost in much the same manner when heterozygous μ-bearers (*Mm*) became homozygous recessives (*mm*). No loss of μ was observed until the 8th cell generation. Thereafter, increasing numbers of cells did not contain μ, and after the 15th generation no cells contained μ.

Further investigations by Gibson and Beale (*29*) led them to propose the following provisional hypothesis:

> The cytoplasm of mate-killer paramecia contains, in addition to the visible μ-particles, certain other factors, here denoted "metagons" which are

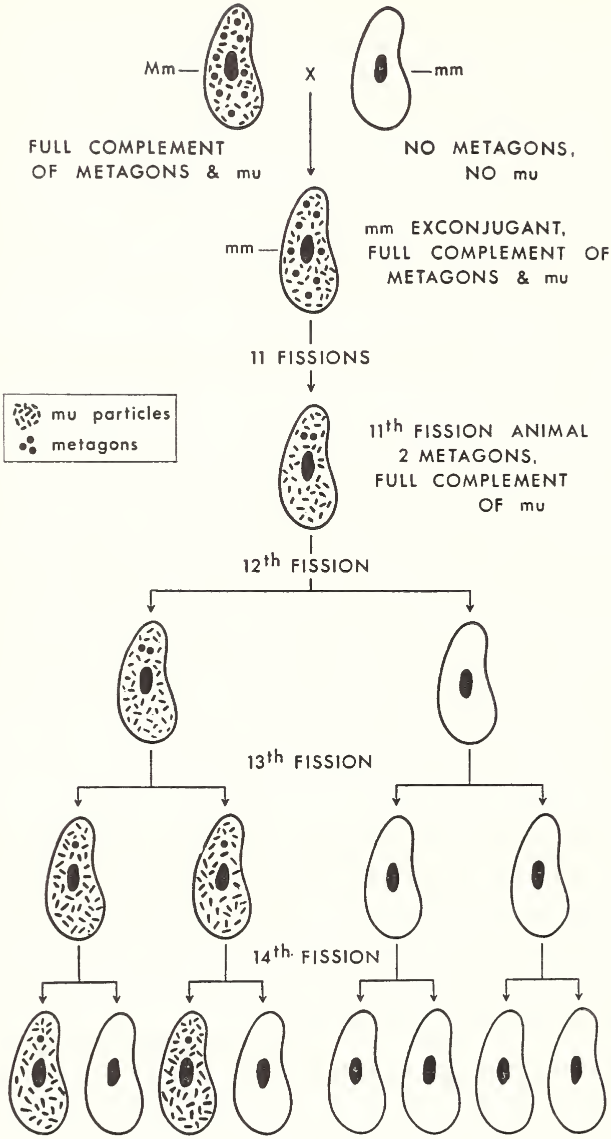
> assumed to be formed only in the presence of one or other of the genes M_1 and M_2. After removal of the dominant genes from a cell by conjugation or autogamy, and subsequent fission of the ex-conjugant or ex-autogamous animals, the metagons would be passively distributed to the daughter cells. Assuming an initial number of 1000 metagons per cell, animals lacking metagons would begin to appear with appreciable frequency after nine fissions, and thereafter the number of animals lacking metagons altogether would rise at each fission. Random fluctuations in the distribution of the metagon amongst the fission products would result in the fluctuation, in different lines, of the stage at which animals without metagons first appear.

Not explicitly stated, but definitely implied, was the assumption that the presence of only *one* metagon in a cell suffices to prevent the loss of a whole population of μ-particles.

On the basis of their experiments, Gibson and Beale postulated that each mate-killer paramecium carried about 10^3 stable nonmultiplying metagons, which were randomly distributed at the time of cell division. After the first seven fissions the random distribution of 10^3 (or 2^{10}) metagons would reduce the number of metagons to a mean of $2^{10}/2^7$ or about 8 per cell. In the following fissions more and more cells with only one metagon or with no metagons would make their appearance. When a cell containing only one metagon would divide, one daughter cell would receive the only available metagon; the other would get none. Both cells would receive roughly half of the μ-particles present in the mother cell. If Gibson and Beale's hypothesis were right, the μ population would increase in the daughter cell which had received the metagon, and the μ population in the metagon-less cell would disappear (text-fig. 10). The results that were obtained were in accordance with the hypothesis. Of the two daughter cells of the 16th generation which arose from the 15th generation μ-bearers, both initially contained μ. One of them retained its μ, but the sister cell lost its μ within 6 hours. Unfortunately, not all the results were as clear-cut. In 5 instances two of the eight 18th generation cells retained μ, and in one instance three of the eight cells retained μ. Disregarding the contradictory results, it was postulated that a single metagon, a nondivisible cytoplasmic unit, can maintain μ.

Going one step further, Gibson and Beale (*30*) reported in 1963 that the metagons could be irreversibly inactivated by treating the paramecia with ribonuclease. Cells bearing μ (*MM*, *Mm*, and *mm*) retained μ after a long exposure (6–12 hours) to high concentrations of ribonuclease (0.5 mg/ml) until after one cell division. All cells lost μ before the second division. Since no effect on the μ-particles could be found (they could be introduced and subsequently maintained into μ-less *M* cells), they postulated that RNA is an essential constituent of the metagon. This led Sonneborn (*31*) to postulate that:

> The simplest and most natural interpretation is that it (ribonuclease) acted on the direct product of gene *M*, that is, messenger RNA. This at once raised the exciting possibility that what has up to this point been called the metagon is actually the m-RNA of gene *M*.


Mm
mm
X
FULL COMPLEMENT OF METAGONS & mu
NO METAGONS, NO mu
mm
mm EXCONJUGANT, FULL COMPLEMENT OF METAGONS & mu
11 FISSIONS
mu particles
metagons
11th FISSION ANIMAL 2 METAGONS, FULL COMPLEMENT OF mu
12th FISSION
13th FISSION
14th FISSION

Text-figure 10.—Metagon hypothesis.

In 1962 Sonneborn cultured a stock of *Didinium nasutum* on stock 51 killer paramecia and found that the didinia had become effective killers of sensitive paramecia, but not of didinia. As long as the didinia were being fed killer paramecia they remained strong killers. When switched to a diet of *kk* paramecia, they were able to maintain κ in their cytoplasm. Further investigations on this phenomenon could not be carried out because this particular strain of *Didinium* was lost.

Gibson and Sonneborn (*32*) fed mate-killer paramecia of stock 540, bearing μ 540 to stock 1 (which at that time was still available) and stock 3 of *Didinium*. Both stocks were apparently able to maintain μ 540 for more than 1,000 cell generations after it ceased to be supplied in the food.

This suggested to Gibson and Sonneborn that *Didinium* itself cannot produce the metagon, but that metagons are acquired from ingested paramecia. They then would replicate indefintely in *Didinium* and as rapidly as the didinia reproduce. Gibson and Sonneborn carried out a series of remarkable experiments. They tried to hybridize RNA extracted from *MM* paramecia (stock 540) possessing metagon activity and from "metagon" carrying didinia with DNA extracted from μ 540 paramecia, *Tetrahymena*, *Aerobacter aerogenes*, and paramecia of genotype *MM* or *mm*. From their results they concluded that the DNA from the *MM* locus is complementary to the RNA of the metagon. The DNA from didinia did not appear to bind any measurable RNA with metagon activity. On this basis it was postulated that the metagon acted as a messenger RNA in paramecia and as a virus in didinia.

Many aspects of the metagon theory are contradictory, unresolved, and nonrepeatable, thus casting doubt on the validity of the concept. Yeung (*33*) could not confirm Chao's basic observations. Instead of being maintained in all cells of a new *kk* clone for 7 or 8 fissions, κ was sometimes lost within 2 fissions. Yeung was not able to find any conditions under which the loss of κ followed the course described by Chao or the course described by Gibson and Beale for the μ-particle. More damaging to the theories proposed by Gibson and Sonneborn (*32*) and Gibson and Beale (*29*) are the findings by Beale and McPhail (*34*) and those by McManamy and Sonneborn (*35*). Beale and McPhail (*34*) report that the disappearance of κ deviates considerably from the metagon-type kinetics. More seriously, McManamy and Sonneborn were *never* able to find loss of either μ or κ after ribonuclease treatment of μ- or κ-bearers. Unpublished experiments by Gibson [*see* Sonneborn (*31*)] state that the abnormally high G + C value of the metagon was due to a "technical difficulty." These discrepancies were thought to be caused by unknown factors, cultural or in the paramecia. I would like to emphasize very strongly that the discrepancies are due to the cultural methods. How can one expect to obtain meaningful results when RNA-DNA hybridization experiments are carried out with RNA and DNA extracted from a mixture of *Didinium*, *Paramecium*, and bacteria?

Not until these experiments are repeated with axenically grown *P. aurelia* (which is possible now) can the claims by Gibson and Sonneborn be verified. There is at present no substantial evidence to support the metagon-mRNA-virus postulate.

DISCUSSION

The biological status of κ and related particles has oscillated between that of contaminating microorganisms, plasmagenes, intracellular parasites, endosymbiotes, proviruses, intermediates between viruses and bacteria, and, as in the case of the metagon, mRNA.

When the killer trait was discovered, Sonneborn (*4*) first suspected that the observed phenomena were due to the presence of a contaminating microorganism. Experiments designed to test this possibility produced only negative results and the hypothesis that the killer trait was due to the presence of another microorganism was abandoned.

Breeding analysis of killers and sensitives of stock 51 of syngen 4 [Sonneborn (*5*)] showed that the killer trait followed the cytoplasm and that the killer and sensitive traits were related to the allelic genes *K* and *k*. This led him to conclude that cytoplasmic factors were a normal part of the genetic system of paramecium. Experiments designed to test Darlington's (*36*) plasmagene theory were seemingly in support of this theory (*6*).

These experiments and others by Sonneborn (*37*, *38*) and Preer (*39*) revealed that κ could be lost irreversibly without apparent harm to the paramecia, and that the amount of κ per cell could increase and decrease, indicating a lack of synchronism between the reproduction of the paramecia and κ. This led Spiegelman, Lindegren, and Altenburg [discussants of (*38*)] to suggest that κ was a symbiotic microorganism. Sonneborn rejected the hypothesis of symbiosis.

When Preer (*7*) in 1948 rendered the κ-particle visible with the Feulgen and acid-Giemsa techniques, and Sonneborn (*40*) in the same year succeeded in infecting nonkillers with κ, Sonneborn (*40*) concluded that the κ system was probably an example of an unusual type of system.

Discussants of κ and its related particles have recognized that many of their features indicate it to be an exogenous particle. These features are: 1) irreversible loss; 2) nonessentiality; 3) nonsynchronous division; 4) random distribution to daughter cells; 5) greater susceptibility to external agents (antibiotics, nitrogen mustard, 2,6-diaminopurine, high temperature); 6) size; 7) infectivity; and 8) reproduction by division.

Sonneborn (*31*) has rejected these eight criteria because neither one of them alone, or a combination of most of them, was according to him a decisive criterion for the existence of exogenous particles within a cell.

Preer and Stark (*41*) described two apparent differences between κ and bacteria: *a*) the failure of techniques which reveal a chromatinic body in bacteria to show such a body in κ; and *b*) the negative response of κ to cytochemical tests for enzymes.

On the basis of the inadequacy of the eight criteria for the exogenous origin of the particles, the DNA distribution as suggested by the Feulgen and acid-Giemsa techniques, and the assumed paucity of enzymes in the κ-particle, Sonneborn (*9*, *42*) postulated that the κ-particle (and the other particles in paramecium) are intermediates between viruses and bacteria.

These points require further comment. The DNA of κ, by cytochemical tests, seems to be distributed throughout the whole particle. Yet, the λ-particle, containing more DNA per unit volume, does not stain with the Feulgen stain. It is clear that cytochemical criteria alone can lead to false conclusions.

Before the availability of suitable axenic media it was impossible to assess whether the particles were nonessential for paramecia. Hence, the rejection of the second criterion by Sonneborn. It must be realized that the large number of nonfastidious bacteria present in the culture medium undoubtedly furnished paramecium with an excess of every nutrilite. The contributions of the endosymbiote to the synthetic machinery of the host would be taken over by the bacteria in the absence of the endosymbiote.

Our experiments established without a doubt that λ is indeed an endosymbiote: 1) It furnishes folic acid to the host, and thus must contain all or part of the enzymatic mechanisms for the synthesis of folic acid; 2) the λ-particle can be grown *in vitro*, and thus must possess the enzymes for synthesizing its cell components; 3) the capability of the λ-particle to synthesize folic acid is reemphasized by the fact that the λ-particles do not require folic acid when grown *in vitro*.

Where does this leave us with the question raised in the introduction: "Can we classify these peculiar cytoplasmic components of *P. aurelia* as neoplastic equivalents?" I believe that the answer can be in the affirmative, as long as we are more broad-minded than Humpty Dumpty.

The essential basis of the difference between killers and sensitives lies in the fact that killers contain large numbers of rod-shaped particles in their cytoplasm. These particles are completely lacking in the cytoplasm of the sensitives. They multiply in the cytoplasm of those strains which carry a certain dominant nuclear gene. They have never been seen to arise *de novo*. Under special conditions the particles can "infect" paramecia possessing the proper genetic background (*40*), even when previously cultured *in vitro* (*21*).

On these grounds the particles are comparable to the viruses associated with certain tumors, especially the milk agent of the mammary tumors of mice, as pointed out by Darlington (*36*), Haddow (*43*), and Heston, Deringer, and Andervont (*44*).

There is yet another analogy I would like to emphasize. Gibor and Granick (*45*) discussed several properties of plastids and mitochondria. There are striking similarities between the inheritable systems of these subcellular components and the cytoplasmic components of paramecium (text-fig. 11). All particles are cytoplasmically inherited, all contain RNA and DNA, all are mutable [for κ *see* Dippell (*46*)], and all are susceptible to antibiotics.

Could it be, perhaps, that these cytoplasmic inclusions found in *P. aurelia*, other protists, and insects represent some very early stage in the evolution of plastids and mitochondria?

It might be suggested that through some unknown mechanism these endosymbiotes escaped digestion in the food vacuoles, and subsequently migrated into the cytoplasm of their host. Here they were established as incipient cell organelles and adapted completely to the environment of their host. They evolved into "a new growth of animal or plant tissue, resembling more or less the tissue from which it arose (or which it invaded), and in the beginning serving no physiological function. They

PROPERTIES	PLASTIDS (Euglena)	MITOCHONDRIA (Yeast)	CYTOPLASMIC PARTICLES (P. Aurelia)
INHERITANCE	.. CYTOPLASMIC ..		
PRESENCE OR ABSENCE OF:			
DNA	.... PRESENT		
RNA	.... PRESENT		
MUTABILITY	.... PRESENT		
SUSCEPTIBILITY TO ANTIBIOTICS	.... PRESENT		

Text-figure 11.—Comparison of the properties of plastids, mitochondria, and cytoplasmic particles.

became virtually identical in value, meaning, or effect with the host tissue." As such they would fall under Webster's definition.

Through evolution these particles finally came to be essential constituents of the cytoplasm of the host, completely dependent on the host for their maintenance, yet maintaining their integrity. In the meantime, the host had become completely dependent on some of the synthetic, energy-trapping and energy-producing mechanisms of their former "neoplastic equivalents."

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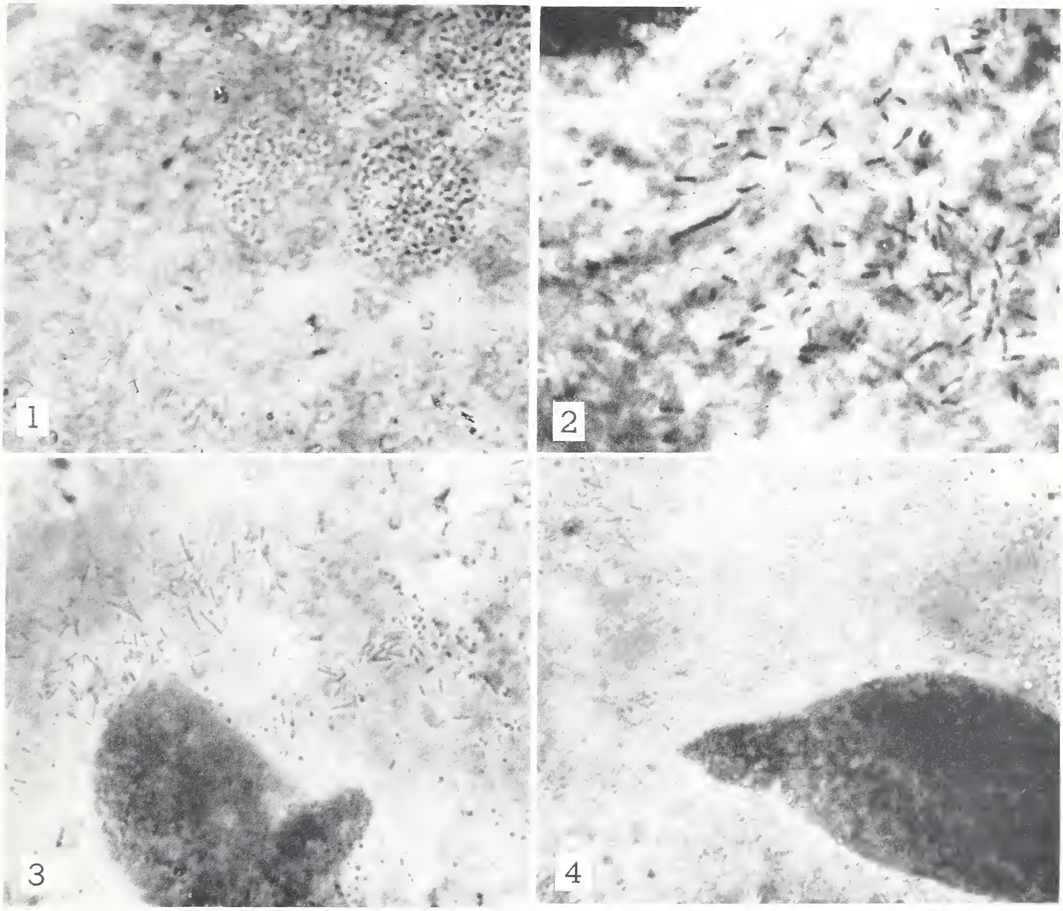
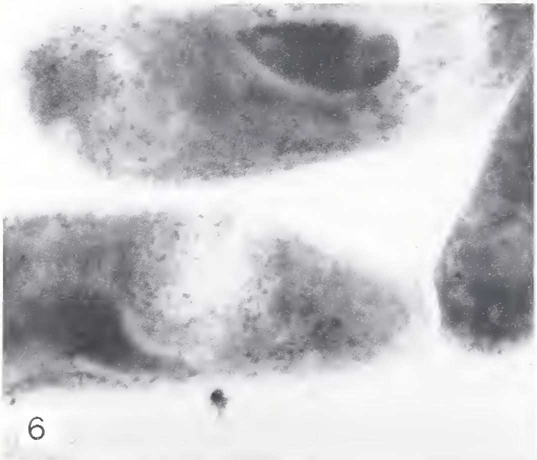


Aceto-Orcein Stain

FIGURE 1.—κ-particle.
FIGURE 2.—λ-particle.
FIGURE 3.—μ-particle.
FIGURE 4.—π-particle.



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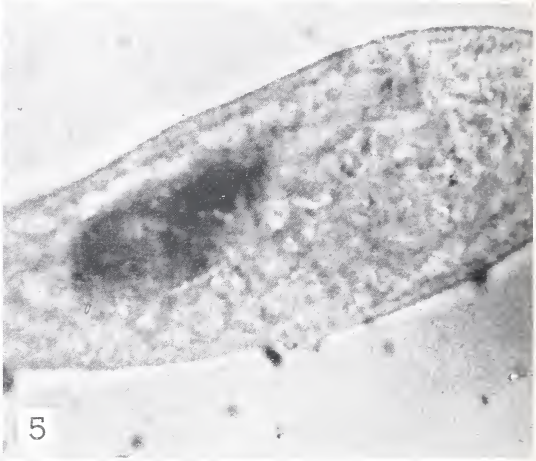

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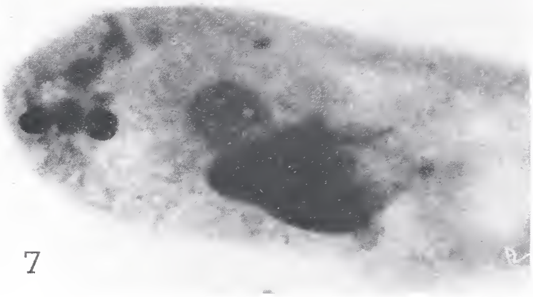

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FIGURE 5.—Feulgen stain of λ-bearing paramecium.

FIGURE 6.—Pyronin Y-Methyl Green stain of λ-bearing paramecium.

FIGURE 7.—Pyronin Y-Methyl Green stain of λ-bearing paramecium after treatment with ribonuclease.

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